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## **Application of Aqueous Biphasic Systems Extraction in Various Biomolecules Separation and Purification: Advancements Brought by Quaternary Systems**

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21 **Title: Application of Aqueous Biphasic Systems Extraction in Various Biomolecules**  
22 **Separation and Purification: Advancements Brought by Quaternary Systems**

23 **Abstract**

24 Aqueous biphasic systems (ABS) extraction is a simple, selective, efficient, and easy to  
25 scale-up technology that, over the years, has attracted a considerable attention from the  
26 researcher community as an alternative methodology in downstream processing of a wide  
27 variety of biomolecules. This review summarizes and discusses the fundamental features of  
28 ABS, as well as its advantages and disadvantages, as a separation and purification  
29 technology of biomolecules. Nevertheless, the focus of this review are quaternary ABS  
30 formed by the addition of neutral salts and ionic liquids to conventional ABS or those ABS  
31 composed by deep eutectic solvents and another phase forming compound. The advantages  
32 brought by quaternary ABS in terms of separation and purification of biomolecules, as well  
33 as the main issues governing the phase behavior of these systems, are discussed. With  
34 examples of application of quaternary ABS as an alternative extraction and purification  
35 methodology, it is shown that such ABS are a promising method to improve the  
36 effectiveness of biomolecules downstream processing, potentially providing a response to  
37 the increasing demand for high purity bioproduct. Furthermore, some of the discussed  
38 quaternary ABS have a great potential as a novel, sustainable and cost-effective purification  
39 platform for biomolecules downstream processing that can potentially simplify the whole  
40 ABS-based purification process due to no need for target bioproduct recovery or phase  
41 formers removal. Finally, perspectives of such quaternary ABS are made, and some future  
42 challenges pointed out.

43 **Keywords:** Aqueous biphasic systems, Additives, Ionic liquids, Deep eutectic solvents,  
44 Downstream processing



## 45 1. Introduction

46 Aqueous biphasic systems (ABS) were discovered in 1896, when Beijerinck observed two-  
47 phase formation upon mixing an aqueous solution of agar and starch or gelatin <sup>[1]</sup>.  
48 Nevertheless, their widespread recognition has begun with the works of Albertsson in mid-  
49 1950s <sup>[2-4]</sup>, where the use of ABS in partitioning of macromolecules, organelles and cells  
50 was explored. In general, ABS consist of two immiscible aqueous-rich phases formed by  
51 mixing two water-soluble substances in exceeding concentrations <sup>[5]</sup> and is described by  
52 binodal phase diagram (see Fig. 1). Hence, these systems can be potentially constructed  
53 with a wide variety of compounds. Nevertheless, the most studied systems are usually  
54 composed of two polymers, a polymer, and a salt or two salts combinations. Moreover,  
55 since both phases of ABS are mainly composed of water, these systems offer a good  
56 environment for separation of biomolecules, without hampering their structural and  
57 biological activities <sup>[6]</sup>. This is the great advantage of this methodology over liquid-liquid  
58 extraction based on organic solvents, where poor solubility and denaturation of the  
59 biomolecules is a common problem <sup>[7]</sup>. Furthermore, ABS-based extraction technologies  
60 are environment-friendly, easy to scale-up, and offer the possibility of continuous operation  
61 mode and integration of several steps into one-unit operation <sup>[8-9]</sup>. Due to those remarkable  
62 features, ABS have been extensively studied as an alternative technology in the extraction  
63 and purification of wide range of biomolecules, such as proteins, enzymes, virus and virus-  
64 like particles (VLPs), among others <sup>[10-18]</sup>.

65 Over the years, ABS have been proven to be advantageous in terms of process economics  
66 and technical simplicity and the idea of using them in the primary recovery and purification  
67 of biological samples was very well received by the scientific community and thereafter the  
68 number of publications regarding this topic significantly increased. Although a lot of studies  
69 have been dedicated to biomolecules partitioning in ABS (see Fig. 2), the mechanisms that

70 rule biomolecule separation within the two coexisting phases are still not fully understood.  
71 Several factors, such as biomolecule hydrophobicity, charge, structure, or size, influence  
72 the preference of the biomolecule to partition to a particular phase of the ABS [5, 10, 15].  
73 Besides biomolecules properties, also ABS features play a crucial role in solute extraction.  
74 Parameters that need to be considered are type, molecular weight and concentration of phase  
75 forming polymer, type, and salt concentration, tie line length (TLL), temperature, pH,  
76 density and viscosity, interfacial tension, and the presence of additives. The influence of  
77 each parameter on partitioning for different ABS was discussed in earlier reviews [10-12, 14-  
78 16]. Therefore, considering the lack of predictive models for ABS selection envisaging a  
79 specific purification problem, considerable number of trial-and-error experiments are  
80 necessary, in order to gain sufficient insights to allow the understanding of the separation  
81 mechanism of each ABS so that high purification factors and recovery yields can be  
82 obtained [19].

83 Moreover, despite the large number of works that showed the potential of ABS extraction  
84 in downstream processing of biomolecules in the batch-scale, until now this methodology  
85 was not adopted in the industrial scale. The main reason of that is not only the poor  
86 understanding of mechanism that governs phase formation and solute partitioning in the  
87 ABS [13], and thus the lack of models that can predict phase splitting behavior, but also the  
88 need for high quantities of chemicals used as phase formers [20], which are sometimes very  
89 expensive (e.g. high molecular weight polymers [21]), additionally increasing the cost of the  
90 process. Thus, throughout the years an effort has been made in order to reduce these  
91 constraints mainly through the development of new cheaper, more efficient, recyclable  
92 phase forming compounds. Introduction of novel phase forming compounds provided new,  
93 interesting schemes for the separation of biomolecules, allowing higher extraction  
94 efficiencies (EE%) and recoveries, as well as the integration of several steps of separation

95 and purification process into one-unit operation <sup>[8-9]</sup>. Additionally, new approaches using  
96 conventional and well-studied ABS were tested. For instance, the addition of a fourth  
97 component to ABS, such as salts or ionic liquids (ILs), has shown to be possible solution  
98 for overcoming the low selectivity (S) of these systems. These additives act as modifiers  
99 and change the properties of the phases in equilibrium, allowing, in many cases, the  
100 complete separation between bioproduct and contaminants. Fig. 3 shows the most important  
101 landmarks in the ABS history since their origin, with the introduction of polymer-polymer  
102 and polymer-salt systems to the first use of deep eutectic solvents (DES) as phase forming  
103 compounds.

104 This paper provides a review on various biomolecules extraction and purification using  
105 quaternary ABS. To the best to our knowledge, this is the first review that discusses entirely  
106 quaternary ABS. The main objective of this review is to discuss the different types of  
107 additives used in formation of quaternary ABS and explain how they can have positive  
108 impact on phase splitting behavior and most importantly on separation and purification of  
109 high-value biomolecules. Furthermore, short introduction to downstream processes for  
110 various biomolecules is made and the need for new cost-effective purification technologies  
111 as part of the bioprocess is highlighted. Finally, examples of application of quaternary ABS  
112 as an alternative extraction and purification methodology are shown. The advancements  
113 made using each type of quaternary ABS are highlighted showing that these systems can  
114 ultimately lead to a powerful technology to purify biomolecules with high quality (high  
115 EE% and/or final bioproduct purity), while being less expensive and safer to the  
116 environment than most conventional ABS, and thus placing them as a promising answer on  
117 the questions raised by the industry on conventional ABS. The perspectives quaternary ABS  
118 are also pointed out, as well as some future challenges.

119



## 120 **2. Additives to conventional ABS: the rise of quaternary ABS**

121 The addition of a fourth component to conventional ABS, composed of polymer-polymer  
122 or polymer-salt, is another approach used to enhance selectivity and to decrease some other  
123 constraints of these systems. In these quaternary ABS one additive (the 4<sup>th</sup> component)  
124 partitions between the two coexisting phases in equilibrium and, consequently, changes  
125 their properties. Very often the use of additives affords lower concentrations of the phase  
126 forming compounds needed to promote two-phase formation. Therefore, more cost-  
127 effective processes can be developed. Furthermore, some additives can act as adjuvants, not  
128 only increasing the separation performance, but also acting as stabilizers for the target  
129 biomolecule. The most extensively used additives are electrolytes (such as NaCl, KCl, KI,  
130 KNO<sub>3</sub>, among others) [22-24], osmolytes (e.g., sucrose, sorbitol, trehalose, urea) [25-26] and ILs  
131 [27]. Moreover, in this section, DES will also be included. Due to the solvation by water of  
132 DES components in ABS and the breaking of the hydrogen bond between hydrogen bond  
133 acceptor (HBA) and hydrogen bond donor (HBD) [28-29], DES components independently  
134 partition between both phases [30-31]. In that way, one of the DES components usually acts as  
135 additive and the other as ABS phase forming compound [30-31]. The strengths and  
136 weaknesses, opportunities and threats of quaternary ABS compared with conventional ABS  
137 are presented in Fig. 4.

138 This section aims to review the most studied families of compounds employed in  
139 development of quaternary ABS. A general discussion on how each group of these additives  
140 changes the ABS properties in terms of biomolecules separation will be provided in the next  
141 subsections. Moreover, special attention will also be given to the effect of the 4<sup>th</sup> component  
142 on the binodal curves of ABS, and some examples will be presented. The name and acronym  
143 of investigated compounds employed in quaternary ABS implementation are listed in Table  
144 1.

145

## 146 **2.1. Electrolytes and osmolytes**

### 147 **2.1.1. The effect of electrolytes and osmolytes on biomolecules partitioning**

148 The addition of electrolytes, such as neutral salts (salts of strong acid and strong base, which  
149 do not hydrolyze and are ionic compounds), are one of the well-known factors which  
150 strongly affect the biomolecule partitioning in polymer-polymer <sup>[22]</sup> and polymer-salt ABS  
151 <sup>[23-24]</sup>. The most widely chosen salt is NaCl, which is considered as being relatively neutral  
152 to a large range of biomolecules. Nevertheless, the concentrations of salts used as additives  
153 are usually kept in a rather low range of concentrations, from 0.0 to 1.0 M, due to  
154 denaturation of proteins in high concentrations of salts <sup>[13]</sup>. In general, the addition of salts  
155 to the conventional ABS increases the ionic strength and the hydrophobic difference due to  
156 generation of an electrical potential variation between the phases <sup>[32]</sup>. An increase in the  
157 hydrophobicity leads to the decrease of the amount of water available for the biomolecule  
158 solvation <sup>[24]</sup>. Therefore, the hydrophobic moieties on the biomolecule surface are exposed  
159 and enhanced partitioning towards the more hydrophobic polymer-rich phase is observed  
160 <sup>[32]</sup>.

161 Another group of compounds used as additives in ABS are osmolytes. They are naturally  
162 occurring compounds found in the cells of many organisms as they help to counteract the  
163 effects of environmental stresses, such as temperature and pH variations, high salinity,  
164 freezing, and dehydration <sup>[33-34]</sup>. There are two types of osmolytes, depending upon their  
165 action on the proteins, protecting and denaturing. Protecting osmolytes have stabilizing  
166 effect on proteins <sup>[35]</sup>, since they bind to the water around the protein surfaces and force  
167 protein folding by excluding water molecules from the protein backbone <sup>[33]</sup>. Different  
168 compounds, such as amino acids (AAs), methylamines, polyols and sugars are representants



169 of protecting osmolytes. On the other hand, denaturing osmolytes, such as urea or guanidine  
170 hydrochloride (GuHCl) <sup>[34]</sup>, bind to the protein backbone and lead to protein unfolding <sup>[33]</sup>.  
171 The observation that protecting osmolytes can force protein to fold without binding to its  
172 backbone indicates that their addition to the solution might change the properties of solvent.  
173 In fact, it has been reported that these molecules change the water structure when in  
174 solutions <sup>[36-38]</sup>. Consequently, the water structure around the biomolecules is controlled by  
175 osmolytes. When added to ABS, they can increase the solute separation by exposing the  
176 hydrophobic groups on the biomolecule surface in similar way to electrolytes. In general,  
177 the partition of biomolecules using ABS in the presence of osmolytes is described as an  
178 effect of changes in the osmolyte-induced solvent properties of aqueous media in the  
179 coexisting phases and not as a direct osmolyte-biomolecule interactions <sup>[25-26]</sup>.

### 180 **2.1.2. The effect of electrolytes and osmolytes on phase equilibrium**

181 In what concerns the effect of salt additives on the binodal curves of ABS, it was shown  
182 that the two-phase area is not significantly affected when compared to the ABS without  
183 additive. In general, the salt addition influences the binodal phase diagrams in a  
184 concentration dependent manner <sup>[39-40]</sup>. It was shown that very small concentrations of salt  
185 additive have no major effect on the shape and position of binodal curves and the higher the  
186 concentration, the more pronounced is the observed effect. Typically, biphasic region  
187 increases with the increasing concentration of salt additive. Moreover, the extent to which  
188 each additive enlarges the biphasic area depends on the nature of the cations and anions of  
189 the salt added <sup>[39-42]</sup>, as well as on the nature of the compounds used to create ABS. In  
190 general, the salts additives composed of the cations and anions with strong salting out ability  
191 (e.g.,  $\text{NH}_4^+$ ,  $\text{K}^+$  or  $\text{SO}_4^{2-}$ ,  $\text{HPO}_4^{2-}$ ) are capable to strongly induce phase formation.





192 Until now, the major focus of the researchers has been the effect of osmolytes on the  
193 partitioning behavior of different solutes in ABS and data on how these compounds affect  
194 phase separation is very scarce. The majority of the works in which binodal phase diagrams  
195 are presented, studies the effect of addition of urea<sup>[43-47]</sup> and guanidine hydrochloride<sup>[45, 48-</sup>  
196 <sup>49]</sup>. To the best of our knowledge there is only one work, which evaluates the effect of amino  
197 acids on the phase behavior of IL-salt ABS<sup>[50]</sup> and one study that evaluates the effect of  
198 glycine, betaine and trimethylamine N-oxide (TMAO)<sup>[47]</sup>. The obtained results showed that  
199 the addition of denaturing osmolytes (urea and GuHCl) to the polymer-salt or polymer-  
200 polymer ABS usually causes the reduction of the biphasic region and, consequently,  
201 increased the concentrations of polymer and salt required to promote phase splitting in  
202 comparison to the same systems without denaturing osmolytes. Furthermore, GuHCl has a  
203 larger influence on the binodal curves than urea, as it decreases the two-phase region to a  
204 greater extent<sup>[45]</sup>. Also, a shift of the binodal towards high concentrations of the phase  
205 forming compounds with the increased concentration of these osmolytes was observed. On  
206 the other hand, data reported for protecting osmolytes, such as AAs, indicate that when they  
207 are present in ABS, they enhance the ability of the liquid–liquid demixing and thus, the  
208 binodal curves become closer to the origin as the concentrations of AAs increase<sup>[50]</sup>. Similar  
209 observation was made in the work of Joshi et al.<sup>[47]</sup> where the addition of protecting  
210 osmolytes (glycine, betaine and TMAO) shifted the binodal curve towards the PEG axis,  
211 most probably due to an improved salting-out ability of the citrate-rich phase in presence of  
212 osmolytes. Furthermore, in this work the authors used conductivity measurements of the  
213 citrate-rich phases to determine the systems tie-lines and tie-lines slope, and compared the  
214 changes in the tie-lines slopes due to the addition of osmolytes to the tie-line slope in  
215 osmolyte-free ABS<sup>[47]</sup>. It was reported that betaine and TMAO protecting osmolytes  
216 decreased the tie-lines slope, while glycine had only slight negative impact as compared to



217 the osmolyte-free system <sup>[47]</sup>. Moreover, the addition of urea resulted in increase of the  
218 slope, but the changes in the final phase compositions were insignificant <sup>[47]</sup>. Overall,  
219 TMAO showed a maximum change in the tie-line slope followed by betaine, glycine, and  
220 urea <sup>[47]</sup>.

## 221 **2.2. Ionic liquids**

222 Another strategy proposed in the formation of quaternary ABS is through the use of ILs as  
223 additives to polymer-polymer or polymer-salt ABS, where they act as adjuvants <sup>[27, 51]</sup> or  
224 electrolytes <sup>[52-53]</sup>. ILs are advanced and highly performant solvents, with unique properties  
225 such as negligible vapor pressure, low flammability, and tunable solvation ability. Along  
226 the years, they have shown to be feasible alternatives to polymer-rich phases <sup>[54-56]</sup> and to  
227 salt-rich phases <sup>[57-58]</sup>. One of the main advantages of ILs-based ABS is the tailoring of their  
228 phase polarities and affinities by a proper manipulation of the cation/anion chemical  
229 structure and their combinations <sup>[27, 59]</sup>. However, some ILs are toxic and expensive, and  
230 this is a major critical issue that have prevented the widespread use of these solvents in  
231 industry. The idea behind the use of ILs as additives in ABS comes from the desire to further  
232 exploit their remarkable properties shown in ABS creation as phase forming compounds  
233 while enhancing the biocompatibility, lowering cost and environmental impact of ILs-based  
234 systems. This crossover between conventional ABS and ILs-based ABS also allowed to  
235 overcome some limitations of polymer-based systems. In particular, polymer-based systems  
236 usually display low selectivity and polarity differences between the two phases, which  
237 greatly affects the purity of the desired product. However, with the wide range of ILs  
238 available and their designer solvent character, it is possible to fine tune the physicochemical  
239 properties of the polymer-rich phase for polymer-polymer and polymer-salt ABS, and by  
240 proper selection of the IL, the extractability and selectivity of a target biomolecule is greatly  
241 improved. The most employed ILs used as additives are imidazolium-based ILs <sup>[27, 51-53, 60-</sup>



242 <sup>69]</sup>. Furthermore, ammonium- <sup>[52, 61, 63-64, 66]</sup>, phosphonium- <sup>[61, 64, 66]</sup>, piperidinium- <sup>[60, 64, 66]</sup>,  
243 pyridinium- <sup>[62]</sup>, pyrrolidinium-based ILs <sup>[60, 62, 64, 66]</sup> or protic ILs <sup>[70]</sup> were also used. The  
244 chemical structures of the most commonly used in ABS IL cations and anions are depicted  
245 in Fig. 5.

### 246 **2.2.1. The effect of ionic liquids on biomolecules partitioning**

247 When ILs are added to ABS, they partition between the coexisting phases and, for the  
248 majority, a preferential partitioning to the polymer-rich phase was observed <sup>[27, 51, 60-61, 64, 66,</sup>  
249 <sup>68]</sup>. Nevertheless, the migration of ILs to a particular phase of ABS depends on the affinity  
250 of ions for water, and the ILs presence in both phases changes their respective physical and  
251 chemical properties <sup>[71]</sup>, thus regulating the extractability of ABS. In general, in such  
252 quaternary ABS, the specific interactions, in particular hydrogen bonds established between  
253 the biomolecules and ILs, play a crucial role in the biomolecules partitioning <sup>[27, 51, 60-66, 68]</sup>.  
254 These specific interactions between biomolecules and ILs are usually more important than  
255 the total amount of ILs present in each phase. Furthermore, besides hydrogen bonding  
256 interactions also the relative hydrophobicity/hydrophilicity of ILs is a crucial property to  
257 manipulate in order to improve system selectivity <sup>[63, 65, 67, 70]</sup>. Thus, as a rule of thumb we  
258 suggest that the selected ILs should have high hydrogen bond basicity ( $\beta$ ) in order to  
259 maximize EE% of the systems. On the other hand, for improved selectivity also the  
260 hydrophobicity/hydrophilicity of ILs should be carefully chosen depending on the nature of  
261 target biomolecule and impurities.

### 262 **2.2.2. The effect of ionic liquids on phase splitting behavior**

263 Apart from the beneficial effect of ILs on the separation and purification performance of  
264 conventional ABS, they have also shown to affect the phase separation ability of the  
265 systems. ILs can either increase or decrease the two-phase region and there is no current



266 understanding on the driving forces of phase formation in such quaternary ABS. This is  
267 probably due to the lack of information of the compositions of the coexisting phases in  
268 quaternary ABS. However, many works state that the ILs cation and anion hydrophobicity,  
269 and thus their affinity for water, affects the phase separation <sup>[27, 72]</sup>. Consequently, the more  
270 hydrophobic ILs usually enhance the two-phase region. Nevertheless, some contradicting  
271 observations have also been made by in our work <sup>[62]</sup> and by Yang et al. <sup>[69]</sup>. In these studies,  
272 the opposite trend was observed and the increase in the ILs hydrophobicity, with increased  
273 IL cation alkyl chain length, enhanced the mutual solubility between the two phases of  
274 polyethylene glycol (PEG) 3350 + (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ABS <sup>[62]</sup> and PEG (800, 1000, 2000) +  
275 Na<sub>2</sub>SO<sub>4</sub> ABS (see Fig. 6 B)) <sup>[69]</sup>. These results oppose those published for PEG (400, 600)  
276 + Na<sub>2</sub>SO<sub>4</sub> (see Fig. 6 A)) <sup>[27, 69]</sup>, where the imidazolium-based IL with the longest alkyl  
277 chain length leads to an enlargement of the two-phase region. These observations clearly  
278 show that beside the ILs properties, also the properties of the phase forming compounds,  
279 such as the nature of salt cation and anion or PEG molar mass, should be taken into account  
280 when discussing the phase behavior of these quaternary systems <sup>[62]</sup>. Furthermore, in the  
281 systems composed of PEG-salt-IL-H<sub>2</sub>O, ILs are enriched in PEG more hydrophobic phase  
282 (as discussed earlier), and thus the interactions between the PEG polymer and ILs also  
283 influence the phase separation behavior. Given these observations, it is possible to find the  
284 most adequate IL adjuvant to a polymer-salt ABS which will result in better performance  
285 in terms of phase separation, requiring lower amounts of each solute to form an ABS. In  
286 that way, comparatively cheaper and more benign IL-based ABS can be afforded due to  
287 lower amounts of IL used.

### 288 **2.3. Deep eutectic solvents**

289 The most recent compounds studied as phase splitters of ABS are DES. DES were  
290 introduced almost two decades ago (in 2003) by Abbott as liquid mixture of two or more



291 compounds resulting from the hydrogen bond interaction of a HBA and a HBD, typically  
292 both solids at room temperature <sup>[73]</sup>. Thus, this liquid mixture exhibits a significantly lower  
293 melting point compared to its pure compounds. When salts are used in the formation of  
294 DES, these solvents share with ILs some of the characteristic features such as high solvation  
295 ability for a large number of compounds <sup>[74]</sup>, similar to that of ILs <sup>[75-76]</sup>. However,  
296 compared with ILs, DES have some advantageous characteristics such as their easier  
297 straightforward preparation, which consists on simply mixing and heating HBDs and HBAs  
298 <sup>[77]</sup>. In addition, the compounds typically used in preparation of DES are abundant,  
299 inexpensive, and very often come from natural sources. Furthermore, the large body of them  
300 can be considered as green solvents with low toxicity <sup>[78]</sup>. Fig. 7 summarizes typical  
301 combinations of HBAs and HBDs used in DES preparation.

302 Owing to these remarkable characteristics, DES were studied in highly diverse fields and  
303 different applications, including ABS implementation, and they are expected to be applied  
304 successfully in large-scale industrial production. The use of DES as phase forming  
305 compounds of ABS was proposed in 2014 by Zeng et al. <sup>[79]</sup> and in recent years more studies  
306 were dedicated to ABS in which DES is used as one of the ABS components. DES  
307 composed of different HBAs and HBDs were used in ABS formation and applied in  
308 extraction and purification of diverse biomolecules, beginning with simple solutes, such as  
309 amino acids, dyes, and ending with more complex molecules such as proteins, enzymes,  
310 nucleic acids or VLPs <sup>[80-81]</sup>. DES have shown to be feasible alternatives to polymer-rich <sup>[28-</sup>  
311 <sup>29]</sup>, salt-rich <sup>[30-31, 79, 82-86]</sup> phases and can also be used in small quantities as adjuvants in  
312 conventional alcohol-salt <sup>[87]</sup> and polymer-salt <sup>[88]</sup> ABS. However, it should be remarked  
313 here, that although the first reports on ABS composed of DES considered them as a new  
314 type of ternary ABS, and a DES-rich and a salt-rich phase were considered to coexist <sup>[79, 84-</sup>  
315 <sup>86]</sup>, in the following years it was shown that such ABS should be regarded as quaternary

316 systems. The reason of that is the solvation of DES components by water leads at large  
317 dilutions to the break of the hydrogen bond between HBA and HBD and the solvation of  
318 the two (or more) independent components <sup>[28-29]</sup>. As a result, when a DES is used in ABS  
319 formulation, we are in fact not dealing with one component aqueous solution but with two  
320 or more solutes depending on the type of DES used. It raises a question whether when  
321 dealing with such ABS they can be named as DES-based ABS because in high dilution  
322 ranges, the DES characteristics no longer exist. In such situation, DES should be considered  
323 as pseudo-component and according to principles for thermophysical and thermochemical  
324 property measurements proposed by Bazyleva et al. <sup>[89]</sup> in phase equilibrium experiments  
325 the components of pseudo-component are expected to be unevenly distributed between  
326 phases and thus pseudo-component should be considered as mixture. Consequently, in ABS  
327 formulation, DES components partition independently to both phases and one of them acts  
328 as an additive and another as phase forming component, this last one enables the  
329 manipulation of the equilibrium and the phases polarities <sup>[30-31]</sup>, and consequently a  
330 conventional ternary ABS is recovered for high dilution of DES. If none of the DES  
331 components have phase splitting ability then the formation of ABS will not be possible.  
332 Nonetheless, in some cases the creation of pseudo-ternary DES-based ABS, where the  
333 initial molar ratio of DES HBA and HBD in both phases in equilibrium is maintained, was  
334 also reported <sup>[28, 82]</sup>. Consequently, in this review, DES are considered as “additives” and  
335 ABS composed of DES and another phase forming compound are regarded as quaternary  
336 systems.

### 337 **2.3.1. The effect of deep eutectic solvents on biomolecules partitioning**

338 All of the above-mentioned findings did not prevent the applicability of ABS composed of  
339 DES in the extraction and purification of biomolecules. It was observed that the partition of  
340 biomolecules is mainly driven by the hydrophobicity difference between the phases and

341 depends on the biomolecule and DES components nature. Furthermore, the concentration  
342 of the DES component which act as an additive influences the partitioning of biomolecules.  
343 In summary, DES-based ABS show high versatility in biomolecules separation and  
344 purification due to large number of possible HBAs and HBDs to be used in DES  
345 preparation. By the change of the nature and molar ratio between HBA and HBD of DES  
346 used in ABS formation, it is possible to tune the properties of the phases and separation of  
347 different biomolecules. Taking all of this into account, when developing new DES-based  
348 ABS, we suggest looking for a DES prepared from starting materials that are commonly  
349 used either as excipients or stabilizers of final bioproduct formulation. The use of such a  
350 DES in ABS, will most likely enable to achieve high EE%, and furthermore will also allow  
351 to avoid the tricky recovery of biomolecule from phase forming compounds. Moreover,  
352 further improvement of EE% can be achieved through manipulation of the ratio between  
353 HBA and HBD since the concentration of the DES component which act as additive has an  
354 impact on biomolecules extraction.

### 355 **2.3.2. The effect of deep eutectic solvents on phase equilibrium**

356 As discussed earlier, ABS composed of DES are quaternary systems, where usually HBD  
357 act as additive and therefore influence the phase properties and biomolecules partitioning.  
358 Consequently, in DES-based ABS the HBA and polymer or salt are responsible for the two-  
359 phase formation and the HBD may or may not impact the ABS formation, depending on its  
360 nature and concentration. For instance, Passos and co-workers studied the effect of the  
361 carboxylic acid nature and concentration on the formation of ABS composed of four DES  
362 (acetic acid:choline chloride (ChCl), glycolic acid:ChCl, lactic acid:ChCl, and citric  
363 acid:ChCl) and polypropylene glycol (PPG) [29]. In general, all DES decreased the binodal  
364 region compared to the systems composed with ChCl and PPG only. It was also stated that  
365 carboxylic acids with small alkyl side chain have a high liquid–liquid demixing ability [29].



366 Furthermore, when the carboxylic acid concentration was increased, the biphasic region  
367 was decreased. However, the representation of binodal curves as a function of the ChCl  
368 concentration revealed that binodal curves are very similar to that of the ChCl without  
369 carboxylic acid (see Fig. 8 A)). This indicates that carboxylic acids have only a minor effect  
370 on the ABS formation, which is mainly driven by salting-out ability of ChCl. Similar results  
371 were reported by Farias et al. for the ABS composed of ChCl:sugars + K<sub>2</sub>HPO<sub>4</sub> + H<sub>2</sub>O (see  
372 Fig. 8 B)), where sugars had also small effect on the formation of DES-salt ABS [31, 83].  
373 However, the opposite results were obtained for ABS composed of ChCl:glucose + PPG +  
374 H<sub>2</sub>O [28] and ChCl:alcohols + K<sub>2</sub>HPO<sub>4</sub> + H<sub>2</sub>O [83]. In these systems, the presence of glucose  
375 and most of the alcohols clearly enlarged the biphasic region in comparison with the system  
376 composed of ChCl alone. As the HBD concentration increased, a lower amount of HBA  
377 (ChCl) was needed to induce the phase separation (see Fig. 8 A) and 8 B)). In the case of  
378 ABS composed of ChCl:glucose + PPG + water, glucose is acting as a salting-out agent  
379 along with ChCl. Furthermore, the authors determined tie-lines and TLLs of such  
380 quaternary systems and it was observed that, independently of the HBA:HBD molar ratio  
381 used with increased TLL, there was a significant decrease of the amount of water and thus  
382 an increase of the ChCl and glucose concentration in ChCl-rich phase [28]. However, the  
383 composition of the PPG-rich phase, composed of more than 76% (w/w) and less than 2%  
384 (w/w) of ChCl and glucose, was not greatly affected by the change of the starting mixture  
385 point[28]. Furthermore, it was observed that the HBA:HBD initial mixture molar ratio was  
386 in close agreement with the ratio measured in ChCl-rich phases [28]. On the other hand, for  
387 PPG-rich phases, the initial molar ratio was not kept for higher HBA:HBD ratio, and when  
388 the molar ratio decreased better results were obtained [28]. On the other hand, in the systems  
389 composed of ChCl:alcohols + K<sub>2</sub>HPO<sub>4</sub> + H<sub>2</sub>O, the formation of an alcohol-rich top phase  
390 and salt-rich bottom phase was observed. The HBA (ChCl) in these systems acts as an





391 adjuvant and is enriched in bottom phase <sup>[30, 83]</sup>. The study on tie-lines and TLLs further  
392 revealed that the HBA:HBD molar ratio of the initial mixture was maintained in the top  
393 phase of ethanol- and 1,2-propanediol-based ABS, while in the bottom phase, due to the  
394 very low concentration of both HBA and HBD, the initial molar ratio was not kept <sup>[30]</sup>.  
395 Moreover, for glycerol-based ABS, due to a high hydrophilic character of glycerol and thus  
396 increased partitioning to the bottom phase, the HBA:HBD molar ratio changed in both  
397 phases for 1:1 and 1:2 mixtures <sup>[30]</sup>. However, at a molar ratio of 1:2 the stoichiometry was  
398 maintained in both phases <sup>[30]</sup>. Furthermore, in case of the n-propanol-based ABS, the  
399 HBA:HBD molar ratio in both phases was totally different of that in initial mixture <sup>[30]</sup>. All  
400 these results indicate that different HBDs lead to different phase equilibria, depending  
401 mostly on HBD nature and concentration. In summary, it can be concluded that the content  
402 and hydrophobicity of HBD, as well as HBA and HBD molecular weights determine phase  
403 splitting behavior <sup>[81]</sup>.

404

### 405 **3. Application of quaternary ABS in biomolecules separation and purification**

406 Due to their advantageous characteristics, ABS have been seen as an alternative, cost-  
407 effective and efficient downstream processing technology, which is suitable for separation  
408 and purification of wide variety of biomolecules. Even though, commonly used systems  
409 composed of polymer-polymer, polymer-salt or ILs showed real advantages in extraction  
410 of biomolecules, very often they were not so effective in terms of bioproduct purity.  
411 Therefore, in order to exploit undeniable potential of conventional ABS as extraction  
412 platform and to further improve its selectivity, an approach in which the properties of the  
413 phase are manipulated through the addition of different additives into the system was  
414 proposed. In that way, quaternary ABS have shown real advantages both in phase separation



415 and purification of biomolecules thus attracting researcher's attention. The advantages of  
416 quaternary ABS over ternary can be especially seen in terms of selectivity and purity levels.  
417 However, it must be mentioned at this point that besides benefits in the systems extraction  
418 and purification efficiency, the addition of fourth compound has made the recovery of  
419 extracted biomolecules and recycling of phase forming compounds more difficult and  
420 complex. The works gathered from literature regarding different quaternary ABS are  
421 discussed below. In the following subsections, the extraction and purification capacity of  
422 ABS for several biomolecules is evaluated through the partition coefficients (K), extraction  
423 efficiencies (EE%) and selectivity (S) values. K is defined as:

$$424 \quad K = \frac{C_T}{C_B} \quad (1)$$

425 where  $C_T$  and  $C_B$  are the concentrations of the biomolecule in the top and bottom phases,  
426 respectively.

427 The extraction efficiency (EE%) is defined according to:

$$428 \quad EE\% = \frac{w_{biomolecule}^T}{w_{biomolecule}^T + w_{biomolecule}^B} \times 100 \quad (2)$$

429 where  $w_{biomolecule}^T$  and  $w_{biomolecule}^B$  are the total weight of biomolecule in the top phase  
430 and in the bottom phase, respectively.

431 The selectivity (S) of target biomolecules compared to the impurity is represented by  
432 equation (3):

$$433 \quad S = \frac{K_{target}}{K_{impurity}} \quad (3)$$

434 Where  $K_{target}$  and  $K_{impurity}$  are the partition coefficients of target biomolecule and major  
435 impurity, respectively.

436 In this section, only biomolecules relevant in food, feed, and pharmaceutical industries,  
437 such as amino acids, proteins, enzymes, monoclonal antibodies and virus or virus-like  
438 particles, will be revised. On top of that, while reviewing the most important achievements  
439 of quaternary ABS in high-value biomolecules partitioning, the focus will be put on  
440 quaternary-based systems formed with ILs and DES.

441 The major reason why ABS technology is widely studied as an alternative method for  
442 separation and purification of biomolecules is the fact that the production step in which the  
443 cellular product is processed to meet purity and quality requirements (downstream process)  
444 is very complex and constitutes the major bottleneck, being a substantial component of total  
445 manufacturing costs.

446 Moreover, most of the currently used methods for separation, concentration and purification  
447 of biomolecules have long processing times, difficulties in scaling-up, among others <sup>[90]</sup>.  
448 Thus, short introduction into the currently used methods in purification of each group of  
449 biomolecules will be provided and the importance of development of new separation and  
450 purification methodology for these biomolecules will be highlighted.

451

### 452 **3.1. Amino acids**

453 Amino acids (AAs) are a very important class of biomolecules as they play an important  
454 role in metabolism, gene expression, signal transduction, and in cellular and extracellular  
455 structures <sup>[91]</sup>. Thus, AAs are essential in animal and human nutrition and they are used in  
456 various applications, such as food additives, feed supplements, components of  
457 pharmaceuticals or sweet taste agents <sup>[92]</sup>. Furthermore, AAs are protein monomers, and  
458 their residues determine the surface properties of proteins. Nevertheless, before they can be  
459 used, AAs must be obtained in high purity levels. That is why downstream processing (DSP)

460 of AAs is a complex process and includes several steps, such as cells removal by  
461 centrifugation and filtration, ion exchange, chromatography, and crystallization [93-94].  
462 Moreover, all these methods are very difficult to scale-up and expensive [95], and thus the  
463 cost related to the AAs downstream processing may account for up to 40% of total  
464 production costs [90]. Therefore, efficient and inexpensive methods for AAs separation,  
465 concentration and purification are in need, so that industrial and society requirements are  
466 met.

467 To overcome such limitations, research focused on ABS and the suitability of this  
468 methodology in extraction of AAs has been widely studied. Moreover, since the details  
469 obtained from the study of single AA partition behavior allow a good understanding of the  
470 driving forces for the partitioning of more complex proteins, AAs have been extensively  
471 used in ABS development as a model biomolecules [11]. The results found in the literature  
472 for the extraction and purification of AAs using quaternary ABS are presented in Table 2.  
473 For example, the effect of salts and osmolytes as additives on the dinitrophenylated amino  
474 acids (DNP-AAs) partitioning in PEG-salt and PEG-dextran ABS was studied by  
475 Zaslavsky's group [26, 96-99]. The authors found out that the presence of salts and osmolytes  
476 affects the properties of the coexisting phases, especially modifying their hydrophobic,  
477 electrostatic differences and the water structure. It was observed that  $K$  of AAs were  
478 affected by the presence of additives in a solute-specific manner. Furthermore, the changes  
479 in  $K$  of DNP-AAs in PEG-salt ABS significantly exceeded those observed for PEG-dextran  
480 ABS.

481 Quaternary ABS composed of ILs have been also employed in various AAs extraction. In  
482 the work of Pereira et al. [27], PEG 600- $\text{Na}_2\text{SO}_4$ - $\text{H}_2\text{O}$  ABS with various imidazolium-based  
483 ILs as adjuvants was used to study the partition of L-tryptophan. The studies revealed that  
484 salting-in inducing ILs increase the  $K$  of L-tryptophan to the PEG-rich phase and in the

485 system where [C<sub>7</sub>H<sub>7</sub>mim]Cl was added an increase in  $K_{\text{Trp}}$  from 20.54 to 42.47 was  
486 obtained. On the other hand, the addition of small amounts of salting-out inducing ILs  
487 decreased the partitioning of this AA. Overall, in this pioneering work was shown that by  
488 adding adequate ILs as adjuvants to the PEG-salt ABS, the L-tryptophan partitioning  
489 behavior can be controlled and manipulated. Afterwards, other AAs such as L-tyrosine [64,  
490 66, 100] and L-phenylalanine [64, 66, 68] were also used as a model biomolecules to test  
491 quaternary ABS composed of different salts, PEGs and ILs. In the works of Coutinho's  
492 group, ABS composed of PEG 400 and weak (potassium citrate) [66] or strong (ammonium  
493 sulfate) [64] salting-out salts with ILs as additives were studied. The partitioning studies  
494 showed that AAs extraction in these systems is probably dominated by differences in  
495 hydrophobicity between the phases. Moreover, the addition of 5% (w/w) of ILs had a small  
496 influence on the AAs extraction. Nevertheless, the results obtained in these works indicate  
497 that the intensity of the IL effect on the partitioning behavior is dependent on the chemical  
498 nature of the salt and the IL used. The use of ILs as additives in conventional ABS can  
499 modulate the extractability according to AAs hydrophobicity. Furthermore, the obtained  
500 results suggest that the weaker salting-out agents allow the enhancement of the IL effect as  
501 additive, not only in terms of tuning the hydrophobicity of the phases, but also by promoting  
502 the occurrence of specific interactions between the ILs and the AAs [64, 66]. The thesis that  
503 the chemical nature of the IL plays important role in AAs partitioning seems to be confirmed  
504 in the work of Hamzehzadeh et al. [100], where an enhancement in the L-tyrosine extraction  
505 into PEG-rich phase of the PEG 600-tripotassium citrate ABS with addition of [C<sub>4</sub>mim]Br  
506 was observed. This increase in K of L-tyrosine when IL was added has been assigned to  
507 specific  $\pi\cdots\pi$  interactions between IL and aromatic AA [100]. These results are in opposition  
508 to what have been noticed for pyridinium and piperidinium ILs in the ABS composed of the



509 PEG 400 and potassium citrate buffer, where the decrease in the  $K$  of L-tyrosine when  
510 compared to the system without IL was observed [66].

511 Amino acids were also used to ascertain the potential applicability of DES-based ABS in  
512 separation and purification of biomolecules. The first study was conducted by Farias et al.  
513 [28] and the ABS composed of PPG 400 and ChCl:glucose DES was employed in extraction  
514 of L-tryptophan, L-phenylalanine and L-tyrosine. The authors showed that the  $K$  have a  
515 clear agreement with the octanol–water partition coefficients for all the AAs studied, with  
516 the exception of L-tryptophan, where specific interactions with ChCl were put forward as  
517 the possible extraction mechanism [28]. All AAs partitioned preferentially to the more  
518 hydrophilic ChCl-rich phase and EE% between 50% and ~90% were obtained.  
519 Furthermore,  $K$  increased with the increase of the TLL. This behavior is related with the  
520 increase of the amount of DES components in ChCl-rich phase, which results in a more  
521 hydrophilic character and consequently higher affinity of the AAs to this phase. On the  
522 other hand, the increase of HBD (glucose) concentration was found to have a small effect  
523 on the  $K$  and only a slight increase of  $K$  was observed, with L-tryptophan partitioning being  
524 the most affected [28]. Later, the same group studied the partitioning behavior of the same  
525 three AAs and glycine now using an ABS composed of ChCl:alcohols DES and  $K_2HPO_4$   
526 [30]. All the AAs, except for glycine, showed high affinity to the top phase, which is mainly  
527 composed of ChCl and alcohol. Furthermore, the  $K$  order agreement with the octanol–water  
528 partition coefficients was observed, with exception of the L-tryptophan, as in the previous  
529 work [28]. Moreover, the effect of the nature and concentration of the alcohol used as HBD  
530 on  $K$  is highly dependent on the AA relative hydrophilic/hydrophobic character. It was  
531 shown that the addition of ethanol results in only slight changes in the  $K$  [30], while di-  
532 alcohols, in particular ethylene glycol, caused a significant decrease in aromatic AAs  
533 extraction and the increase in aliphatic glycine partitioning to the top phase, due to



534 hydrophilic character of di-alcohols. Additionally, the system composed with ChCl:1,2-  
535 propanediol (1:1) DES was shown to be highly effective in the separation of aromatic AAs,  
536 such as L-tryptophan and L-tyrosine, and the selectivity ( $S_{\text{Trp/Tyr}}$ ) of 30.9 was obtained,  
537 highlighting the potential of fine HBD tuning for better separation performance. More  
538 recently, Chao et al. <sup>[101]</sup> showed the potential of DES-salt ABS in extraction of L-  
539 tryptophan. The DES composed of ChCl and PEG 2000 (mass ratio of 2:3) was combined  
540 with different salts, namely  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ ,  $\text{Na}_2\text{CO}_3$ ,  $\text{NaH}_2\text{PO}_4$ , or  $\text{K}_2\text{HPO}_4$  <sup>[101]</sup>. These systems  
541 were shown to be highly efficient in this AA extraction and the amount of DES, as well as  
542 the salting-out ability of the salt, were the main factors that affected the extraction. Overall,  
543 after optimization, EE% of 93.88%, 90.83%, 88.88% and 86.72% for ChCl:PEG 2000 (2:3)-  
544  $\text{K}_2\text{HPO}_4$ , DES- $\text{NaH}_2\text{PO}_4$ , DES- $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$  and DES- $\text{Na}_2\text{CO}_3$  ABS were obtained,  
545 respectively <sup>[101]</sup>.

### 546 **3.2. Enzymes**

547 While most of the commercial applications of enzymes do not require high purity of the  
548 final bioproduct, for their usage in food, cosmetics or pharmaceutical industries, purified  
549 enzymes preparations are compulsory <sup>[102]</sup>. There are many conventional purification  
550 methods used for recovery and purification of enzymes. They include ammonium sulfate  
551 precipitation, ultrafiltration and different chromatography such as size-exclusion, ion  
552 exchange, hydrophobic interaction and affinity chromatography; or some combinations of  
553 these techniques <sup>[103]</sup>. However, all these methods do not meet the industrial requirements  
554 because they are time-consuming and expensive. For instance, ultrafiltration and  
555 hydrophobic interaction chromatography consist of several steps. Moreover, in affinity and  
556 ion exchange chromatography, which are commonly used in the purification of recombinant  
557 enzymes, a sample pretreatment step is required. As a result, at each step of these multi-step  
558 protocols, some quantity of the target enzyme is lost and, thus, low final recovery yields are



559 achieved. Since conventional liquid-liquid extraction based on organic solvents are  
560 generally not suitable for the purification of enzymes, due to the irreversible loss of  
561 enzymatic activity <sup>[104]</sup>, the research focus has been ABS. ABS were proposed for  
562 purification of enzymes as an alternative methodology able to overcome the before  
563 mentioned limitations, while being cost-effective, efficient and capable of combining  
564 several steps of different downstream processes into one-unit operation <sup>[104-106]</sup>.

565 A summary of quaternary ABS found in the literature for the extraction of enzymes is  
566 presented in Table 3.

567 The effect of different salts used as additives in ABS has been largely studied since salts  
568 can modify the enzymes partition to one of the phases, changing purification factors and  
569 recovery yields, without hampering their activity. For example, in the work of Ooi et al.  
570 <sup>[107]</sup>, the addition of 4.5% (w/v) of NaCl to 2-propanol-K<sub>2</sub>HPO<sub>4</sub> ABS increased the lipase  
571 recovery from 76% to 99% and purity from 6.4 to 13.5-fold in alcohol-rich phase. In another  
572 study, conducted by Barbosa and co-workers, although the addition of NaCl did not  
573 significantly improve K of lipase, the purification factor increased from 59.93 to 141.65  
574 fold when 6% (w/v) NaCl was added to the PEG 8000-potassium phosphate ABS <sup>[108]</sup>.  
575 However, the highest concentration of NaCl added, decreased the K of lipase <sup>[108]</sup>. Similar  
576 trend of improved recovery and/or purity was also observed for other enzymes. The  
577 recovery of protease from *Calotropis procera* in PEG-rich phase was significantly  
578 enhanced, from 23.58% to 107%, with addition of 6% (w/w) NaCl to PEG 4000-MgSO<sub>4</sub>  
579 <sup>[109]</sup>. Furthermore, increased recovery of invertase and increased purity in PEG-rich phase,  
580 from 68% to 90% and 3.3 to 5.5-fold, respectively, were observed when 5% (w/w) KCl was  
581 added to PEG 3000-Na<sub>2</sub>SO<sub>4</sub> ABS <sup>[110]</sup>. The addition of 4.5% (w/w) NaCl also allowed to  
582 obtained higher K (84.2), purification factor (14.37) and yield (97.3%) of serine protease  
583 from mango peel in PEG-rich phase of PEG-dextran ABS<sup>[111]</sup>. In general, it is evident that





584 by adding neutral salts to the conventional ABS, improved extraction performance for the  
585 target enzymes is achieved. Moreover, very often the changes in phase properties induced  
586 by added salt may also result in increased enzyme recovery and purification factor.

587 The strategy of using ILs as adjuvants in ABS for improved purification of enzymes was  
588 also ascertained for lipase <sup>[67]</sup> and L-asparaginase <sup>[65]</sup>. The effect of 5% (w/w) of  
589 imidazolium-based ILs on lipase from *Bacillus* sp. ITP-001 purification in ABS composed  
590 of different PEGs and K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> was first studied. After the optimization procedure,  
591 in the PEG 1500-K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> ABS, with [C<sub>6</sub>mim]Cl added, the purification factor  
592 increased from 175.61 (no IL added) to 245 <sup>[67]</sup>. This high increase in purity was a result of  
593 the favorable interactions between the IL and the contaminant proteins. The presence of  
594 [C<sub>6</sub>mim]Cl greatly improved the partition of contaminant proteins to the PEG-rich phase,  
595 also enriched in IL, while the lipase remained in the salt-rich phase. On the other hand, the  
596 favorable effect of ILs on L-asparaginase purification performance of PEG-citrate buffer  
597 ABS was also observed by Santos et al. <sup>[65]</sup>. High recoveries of 87.94%, purification factors  
598 of 20.09 and a final specific activity of 3.61 U/mg were observed for ABS composed of  
599 PEG 6000 and citrate buffer with 5% (w/w) of [C<sub>4</sub>mim][CH<sub>3</sub>SO<sub>3</sub>] <sup>[65]</sup>. Due to high affinity  
600 of L-asparaginase to the PEG-rich phase, ILs that display low polarity and hydrogen bond  
601 basicity, such as [C<sub>4</sub>mim][CH<sub>3</sub>SO<sub>3</sub>] and [C<sub>4</sub>mim][CF<sub>3</sub>SO<sub>3</sub>], were not capable to establish  
602 strong specific interactions with the contaminant proteins and, as a consequence, to improve  
603 the partitioning of these proteins in salt-rich phase. Furthermore, ILs with high hydrogen  
604 bond basicity decreased the L-asparaginase purification factor since they promoted the  
605 specific polar interactions between contaminant proteins and ILs in the top phase. The  
606 results obtained in those works, indicate that by proper selection of the IL anion it is possible  
607 to manipulate the partitioning behavior of contaminant proteins and thus allowing to  
608 increase the purification performance of the PEG-salt ABS for two different enzymes.



609 DES-based quaternary ABS were used, for the first time, in extraction of a model enzyme  
610 (trypsin) in the work conducted by Zeng and co-workers <sup>[79]</sup>. In this study, DES composed  
611 of ChCl: urea (1:2) ChCl:methylurea (1:2), [N<sub>1111</sub>]Cl:urea (1:2) and [N<sub>3333</sub>]Br:urea (1:2)  
612 were used to create ABS with K<sub>2</sub>HPO<sub>4</sub> salt. The highest EE% in DES enriched phase of  
613 95.53% was obtained in the system containing ChCl:urea DES. Moreover, slightly lower  
614 EE% (95.27%) was achieved using ChCl:methylurea DES, suggesting that the nature of the  
615 HBD does not have a major impact on the trypsin partitioning <sup>[79]</sup>. On the other hand, HBA  
616 nature seems to play a crucial role in the extraction of the trypsin since when [N<sub>1111</sub>]Cl and  
617 [N<sub>3333</sub>]Br were used, a significant decrease in EE% was registered, 81.31% and 36.87%,  
618 respectively <sup>[79]</sup>. Trypsin was also used while evaluating the extraction potential of betaine-  
619 based DES ABS <sup>[112]</sup>. Contrary to the previous work <sup>[79]</sup>, it was shown that the HBD nature  
620 greatly affects the EE% of trypsin. Finally, a high EE% of 94.36% in DES enriched phase  
621 was achieved for trypsin in ABS composed of ChCl:glycerol and K<sub>2</sub>HPO<sub>4</sub> <sup>[113]</sup>. Moreover,  
622 the extraction of papain was studied using ABS composed of ChCl:PEG 2000 DES and  
623 NaH<sub>2</sub>PO<sub>4</sub>, Na<sub>2</sub>CO<sub>3</sub>, Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub> salts <sup>[114]</sup>. The papain EE% in DES-enriched phase  
624 decreased according to the order of phase forming ability of the salt: Na<sub>2</sub>CO<sub>3</sub> > Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>  
625 > NaH<sub>2</sub>PO<sub>4</sub>. The molar ratio between ChCl and PEG 2000 was shown to highly affect the  
626 enzyme partitioning. It was observed that using the ChCl:PEG 2000 (20:1), the EE%  
627 increased from 54.42% (molar ratio (1:1)) to 83.50% <sup>[114]</sup> and after optimization 90.95%  
628 efficiency was attained <sup>[114]</sup>. Furthermore, in our work the extraction of pepsin using DES-  
629 based quaternary ABS was studied <sup>[115]</sup>. We used ABS composed of PPG 425 and DES  
630 prepared with betaine hydrochloride (BeHCl) as HBA and fructose, glucose, sucrose, urea  
631 as HBDs. Although pepsin extraction studies showed a high affinity of this enzyme to the  
632 BeHCl/DES-rich phase, the ternary ABS composed of BeHCl and PPG 425 was the best  
633 system in terms of pepsin EE%, with 99.8% <sup>[115]</sup>. However, when glucose was used as HBD,



634 an increased recovery activity of 141.9% and EE% of 99.5% were attained <sup>[115]</sup>. Therefore,  
635 the presence of adequate HBD might lead to specific interactions between an enzyme and  
636 HBD that yield an increased activity of pepsin.

### 637 **3.3. Monoclonal antibodies**

638 Antibodies are glycoproteins present in plasma and extracellular fluids that have binding  
639 specificity for particular antigens <sup>[116]</sup>. Nowadays, antibody-based therapies play an  
640 important role in the treatment of many infectious diseases, autoimmune disorders, and  
641 cancers. Therefore, the demand for antibodies is constantly increasing. However, to use  
642 antibodies as therapeutic agents, their production has to meet high safety standards and high  
643 levels of purity in the final product <sup>[117]</sup>. Although, improvements have been made in both  
644 upstream and downstream processes of antibodies, the high cost of the currently used  
645 purification methods is still the major bottleneck that has been preventing the widespread  
646 use of these biopharmaceuticals. Typical downstream processing of antibodies is composed  
647 of several steps, which include (i) clarification by removal of cells and cell debris by  
648 centrifugation or microfiltration, (ii) concentration by ultrafiltration, (iii) purification by  
649 chromatography, (iv) virus inactivation and removal and (v) validation and quality control  
650 tests <sup>[118]</sup>. Furthermore, the purification step can account for up to 90% of the total  
651 downstream costs and the whole downstream processing is responsible for 50-70% of total  
652 production costs <sup>[90]</sup>. The major reason for that is the fact that in the purification step very  
653 expensive chromatographic methods are usually used, which are also very difficult to scale-  
654 up. Thus, in order to solve these shortcomings, the separation and purification using ABS  
655 has been proposed. However, the applicability of ABS in downstream processing of  
656 antibodies so far has been limited to the academic studies only <sup>[24]</sup>.



657 The results found in literature for the extraction and purification of monoclonal antibodies  
658 using quaternary ABS are presented in Table 4.

659 The first report, in which the use of salt as additive to ABS for purification of antibodies,  
660 dates back to 1996 <sup>[119]</sup>. In this work, Andrews and co-workers employed PEG 1450-  
661 phosphate system for the purification of murine immunoglobulin G (IgG) from a hybridoma  
662 supernatant. The developed strategy consisted of two steps - extraction and back extraction.  
663 In the extraction step, the addition of 12% (w/w) NaCl promoted the antibody partitioning  
664 to the PEG-rich phase, while the impurities preferentially concentrated in the salt-rich phase  
665 <sup>[119]</sup>. The antibodies were then successfully back-extracted using the same ABS but formed  
666 with fresh phosphate solution without NaCl. Overall, at the end of the process, IgG purity  
667 of 80%, 5.9 purification fold and 100% recovery, were achieved <sup>[119]</sup>. Over a decade later,  
668 Aires-Barros group brought back the idea of using ABS with NaCl as additive for the  
669 purification of antibodies in a series of articles <sup>[24, 117-118, 120-123]</sup>. At first, the authors used  
670 PEG-phosphate systems for the purification of antibodies from an artificial mixture of  
671 proteins composed of human serum albumin and myoglobin <sup>[118]</sup>. The same trend of  
672 increased antibodies partitioning towards the PEG-rich phase, decreasing the impurities  
673 with increased NaCl concentrations was observed, corroborating Andrews et al. results <sup>[119]</sup>.  
674 The highest purification was obtained using the ABS composed of PEG 3350, phosphate at  
675 pH 6 and 15% (w/w) NaCl with a recovery yield of 101%, a purity of 99% and a yield of  
676 native IgG of 97% <sup>[118]</sup>. Later on, the same strategy was applied in purification of antibodies  
677 from real matrices, such as Chinese hamster ovary (CHO) and hybridoma cell supernatants  
678 <sup>[117]</sup>. Once again, it was shown that high concentrations of NaCl maximize the partition of  
679 IgG into the top PEG-rich phase of the PEG 6000-phosphate buffer pH 6 ABS, while the  
680 impurities were mostly found in the bottom phase <sup>[117]</sup>. As a result, recovery yield of 88%  
681 and 90% in a PEG-rich phase and a purification factor of 4.3 and 4.1 for IgG from CHO



682 and a hybridoma cell culture supernatants were obtained, respectively <sup>[117]</sup>. Later, the  
683 phosphate salt was replaced by the biodegradable citrate salt in order to decrease the  
684 environmental impact of these ABS. As observed for PEG–phosphate systems, an increase  
685 in the concentration of NaCl favored the IgG partitioning to the top phase <sup>[120]</sup> and, by  
686 changing the concentration of NaCl, it was also possible to manipulate the IgG partitioning  
687 behavior towards the phase with fewer impurities, also in the PEG–citrate ABS. Moreover,  
688 in the same year (2009), the same group proposed a multi-stage approach using PEG-  
689 phosphate ABS containing 10% (w/w) NaCl ABS <sup>[24]</sup>. The authors showed that, by using  
690 multi-stage extraction, higher IgG recovery and purity than in single-stage experiment can  
691 be obtained, as 89 % of IgG with 75% purity was recovered in PEG-rich multi-stage  
692 extraction, while only 61% recovery and 55% purity were achieved in single-stage  
693 extraction <sup>[24]</sup>. In the following years, Aires-Barros’s group focused on the possibility of  
694 integration of ABS extraction with NaCl as additive in downstream processing of  
695 antibodies. The continuous extraction using packed differential contactor was evaluated in  
696 the purification of human IgG from CHO cells supernatant, using the PEG 3350-phosphate-  
697 NaCl ABS <sup>[121]</sup>. The PEG-rich phase was continuously dispersed at the bottom of the  
698 column through a capillar and the phosphate-rich phase was continuously fed at the top of  
699 the column <sup>[121]</sup>. In this continuous ABS extraction set-up, an improved IgG recovery yield  
700 of 85% and a purification factor of 1.84 were obtained compared to the batch extraction,  
701 where recovery yield of 61% and purification factor of 1.59 were observed <sup>[121]</sup>. Moreover,  
702 the continuous-flow process in microfluidic device for the extraction of mAbs in the ABS  
703 composed of PEG-phosphate with NaCl as additive was also proposed by Silva et al. <sup>[123]</sup>.  
704 A fluorescently tagged IgG was used in the partition studies and the results obtained in this  
705 microscale were found to be in agreement with those obtained in batch laboratory scale,  
706 while reducing the operation time and allowing the continuous monitoring of the separation



707 process <sup>[123]</sup>. Finally, an attempt to replace the low capacity, difficult to scale-up and  
708 expensive chromatography by a continuous ABS extraction process, has been made by Rosa  
709 et al. <sup>[122]</sup>. In this study, a continuous process incorporating three different steps (extraction,  
710 back-extraction, and washing) was set up and validated in a pump mixer-settler battery.  
711 ABS composed of PEG 3350-phosphate buffer at pH 6 with NaCl was used and the IgG  
712 from CHO and PER.C6® cell supernatant was purified. The removal of the high molecular  
713 weight impurities was observed in the extraction step and the back-extraction and washing  
714 allowed further IgG purification and separation from the lower molecular weight impurities  
715 and polymer-rich phase, as well as the PEG recycling <sup>[122]</sup>. Overall, IgG recovery yield of  
716 80% and a final total purity of 97% from CHO supernatants was achieved. Furthermore,  
717 100% recovery yield with a promising host cell protein/IgG ratio was observed for IgG  
718 purification from PER.C6® cell supernatant <sup>[122]</sup>. All these studies clearly show that  
719 quaternary ABS, composed of PEG, citrate salt and NaCl as fourth component, constitute  
720 an economical and benign alternative methodology for the purification of monoclonal  
721 antibodies.

722 In another vein, polymer-salt ABS, this time using ILs as adjuvants, were also evaluated in  
723 extraction and purification of IgG from rabbit serum samples <sup>[61]</sup>. After optimization, the  
724 ABS composed of PEG 400 and citrate buffer at pH 7 was selected and 5% (w/w) of  
725 different ILs added. Imidazolium-based, quaternary ammonium and phosphonium ILs were  
726 selected allowing the evaluation of the IL cation and anion nature effects, as well as the  
727 effect of increase of the alkyl side chain length. The addition of the 5% (w/w) of ILs that  
728 combined imidazolium cation and [CH<sub>3</sub>CO<sub>2</sub>]<sup>-</sup>, Cl<sup>-</sup>, and [TOS]<sup>-</sup> anions resulted in an increase  
729 IgG EE% from 96% (with no IL added) to 100% in a single-step <sup>[61]</sup>. It was concluded that  
730 specific hydrogen-bonding and  $\pi\cdots\pi$  interactions (in the case of [C<sub>4</sub>mim][TOS]) play a  
731 crucial role in the improved IgG extraction to the PEG-rich phase. The ILs with anions with



732 a higher hydrogen bond basicity were able to induce complete extraction of IgG, while this  
733 was not attained with the ILs that display lower hydrogen bond basicity, such as [C<sub>4</sub>mim]Br  
734 and [C<sub>4</sub>mim][N(CN)<sub>2</sub>]. Furthermore, quaternary ammonium- and quaternary  
735 phosphonium-based ILs also led to the complete extraction of IgG to the polymer-rich phase  
736 and no major differences on the IgG partitioning as a function of the alkyl chains length of  
737 these ILs was observed <sup>[61]</sup>. The same observation was made for the alkyl side chain length  
738 effect of imidazolium-based IL, thus indicating that no significant interactions between the  
739 IL cations and the protein surface take place in these systems <sup>[61]</sup>. Moreover, the  
740 advantageous performance of the quaternary ABS with ILs was also confirmed in IgG  
741 purification from rabbit serum samples, and the complete extraction and an enhancement of  
742 ca. 37% in the IgG purity was obtained by the use of [C<sub>4</sub>mim][CH<sub>3</sub>CO<sub>2</sub>] as adjuvant <sup>[61]</sup>.  
743 This work shows that the addition of small quantities of ILs to the polymer-salt ABS  
744 constitute a viable and scalable strategy to extract and purify antibodies from real serum  
745 samples to be used as therapeutic agents. Freire's group also studied the effect of addition  
746 of chloride-based ILs to PEG 6000-dextran ABS on partitioning of IgG <sup>[124]</sup>. It was revealed  
747 that the partition coefficients increased in following order: [P<sub>4444</sub>]Cl < no  
748 IL  $\approx$  [C<sub>4</sub>mim]Cl  $\approx$  [C<sub>4</sub>mpyr]Cl < [N<sub>4444</sub>]Cl < [C<sub>4</sub>mpip]Cl, and that in all systems IgG preferred  
749 dextran-rich phase ( $K < 1$ ) <sup>[124]</sup>. Moreover, 5% (w/w) of [C<sub>4</sub>mpip]Cl and [N<sub>4444</sub>]Cl and 10%  
750 (w/w) of [C<sub>4</sub>mpip]Cl and [C<sub>4</sub>mim]Cl favored IgG migration to the PEG-rich phase mainly  
751 as an effect of IL-IgG interactions depending on the chemical structure of the IL <sup>[124]</sup>.  
752 Furthermore, improved selectivity between Cyt C and IgG was obtained with the addition  
753 of 5% (w/w) of [C<sub>4</sub>mpyr]Cl, [N<sub>4444</sub>]Cl, and [P<sub>4444</sub>]Cl compared to the ABS without IL <sup>[124]</sup>.  
754 Also, higher selectivity values between BSA and IgG, compared with the ABS without IL,  
755 were observed using 5 % (w/w) of [C<sub>4</sub>mim]Cl and [C<sub>4</sub>mpip]Cl <sup>[124]</sup>. Overall, these results



756 further highlight that using ILs as adjuvants constitute viable approach to improve  
757 extraction and purification of antibodies.

758 Even though the high potential of quaternary ABS in extraction and purification of  
759 antibodies have been described using salts and ILs additives, at the moment there is no  
760 reports evaluating the use of DES-based ABS in the separation and purification of these  
761 therapeutic agents. The reason is most probably the fact that DES as phase former  
762 compounds of ABS were introduced only in 2014 and consequently only the extraction of  
763 model biomolecules such as amino acids, proteins, alkaloids, among others, were studied.  
764 Their true potential in purification of monoclonal antibodies still remains to be proved.

#### 765 **3.4. Proteins**

766 Proteins are larger biomolecules that consist of long chains of amino acid residues. They  
767 are present in all living organisms, where they play a vast array of functions. Therefore,  
768 proteins have been applied in various industries, such as in food, feed and pharmaceuticals  
769 sectors <sup>[125]</sup>. Downstream processing of proteins consists of several steps and various unit  
770 operations due to complexity of starting material <sup>[126]</sup>. In order to meet the regulatory  
771 requirements for purity and quality of proteins for pharmaceutical or food applications, both  
772 low- and high-resolution technologies are used. They include tangential flow filtration,  
773 ultra/diafiltration and different chromatography methods (IEC, SEC, HIC, RP, multimodal  
774 and affinity). Furthermore, mild operation conditions for their recovery and purification are  
775 necessary because proteins can be very easily denatured and lose their native structure and  
776 function <sup>[125]</sup>. Chromatography of proteins is very often considered as not scalable and an  
777 expensive methodology and, in the large scale, some difficulties such as slow protein  
778 diffusion or discontinuity in the process are observed <sup>[126]</sup>. Moreover, the high cost of protein  
779 bioseparation continues to remain a major drawback in manufacturing of proteins, with up





780 to 80% of total bioprocessing costs for plasma proteins <sup>[90]</sup>. Therefore, the development of  
781 cost-effective and high yielding purification methods for proteins is still an unmet  
782 challenge. To overcome these shortcomings, research has been focused on ABS and the  
783 quaternary ABS formed with salts, osmolytes, ILs or DES have been shown to be beneficial  
784 in the separation of proteins.

785 A summary of results found in literature for the extraction and purification of proteins using  
786 quaternary ABS are presented in Table 5.

787 The effect of addition of different salts to PEG-dextran and PEG-phosphate ABS was  
788 studied by Cascone et al. <sup>[127]</sup> for the partitioning and purification of thaumatin. Thaumatin  
789 is a protein sweetener used as flavor and aroma enhancer <sup>[128]</sup>. The obtained results showed  
790 that, using NaCl in PEG 6000-phosphate ABS, K of thaumatin significantly increased from  
791 0.53 to 33 when 1.5 M of NaCl was added to the system <sup>[127]</sup>. Moreover, this effect was  
792 more pronounced in PEG-phosphate ABS than in PEG-dextran systems. Much lower  
793 improvements in the K were found for other salts, such as  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{NaClO}_4$  <sup>[127]</sup>.  
794 Therefore, the purification of thaumatin from *E. coli* homogenate proteins and BSA was  
795 conducted using PEG-phosphate-NaCl ABS. It was shown that K of homogenate proteins  
796 and BSA decreases with the addition of NaCl to the systems and the observations made  
797 using individual proteins were maintained for the mixture of thaumatin and *E. coli*  
798 homogenate proteins, with 90-95% recovery yield and a 20-fold purification in one step  
799 <sup>[127]</sup>. The significant increase of the K by the addition of NaCl to PEG-salt systems seems  
800 to significantly affect the protein's hydrophobicity <sup>[32]</sup>. The authors observed that the  
801 addition of NaCl to PEG-phosphate ABS increases the hydrophobic difference between the  
802 phases and promotes hydrophobic interaction between the proteins (BSA, lysozyme,  
803 conalbumin,  $\alpha$ -lactalbumin and  $\alpha$ -lactoglobulin A) and PEG <sup>[32]</sup>. This fact was further  
804 confirmed in the work of Franco et al. <sup>[52]</sup>, where two different series of hydrophobically

805 modified proteins were partitioned in PEG-phosphate ABS with addition of NaCl <sup>[129]</sup>. The  
806 authors observed an increase in separation capacity of these systems, when compared with  
807 the same systems without NaCl <sup>[129]</sup>. Moreover, Fan and Glatz reported that T4 lysozyme  
808 partitioning in PEG-dextran ABS with salt additives shifts more protein from the bottom to  
809 top phase. The authors also observed that the different concentrations of salt also influence  
810 electrostatic and non-electrostatic interactions <sup>[130]</sup>. Furthermore, the addition of different  
811 concentrations of NaCl (0.0-1.0 M) increased the K of myoglobin and ovalbumin from 4.20  
812 to 15.77 and 2.82 to 5.51, respectively, in the PEG 4000- polyacrylic acid (PAA) ABS <sup>[20]</sup>.

813 In similar context, Zaslavsky's group conducted several studies on the effect of osmolytes  
814 on the partitioning of 11 different proteins, namely trypsinogen,  $\alpha$ -chymotrypsinogen A,  
815 ribonuclease A, ribonuclease B,  $\beta$ -lactoglobulin A,  $\beta$ -lactoglobulin B, papain,  
816 chymotrypsin, lysozyme, hemoglobin, and concanavalin in PEG-dextran and PEG-  
817 phosphate buffer (0.01 M phosphate buffer, pH 7.4) ABS <sup>[25, 99, 131]</sup>. They used sorbitol,  
818 sucrose, trehalose, and TMAO as additives. It was shown that differences in the K for  
819 proteins in the presence of 0.5 M of different osmolytes were exclusively related to solvent  
820 properties of the coexisting phase, with no direct interaction between the osmolytes and the  
821 proteins <sup>[25]</sup>. The authors also concluded that proteins responses to the presence of different  
822 osmolytes in ABS are governed by the proteins structures and that these effects are less  
823 pronounced than those observed in the presence of salts additives <sup>[99]</sup>.

824 The first reports on the use of ILs as additives to the polymer-polymer or polymer-salt ABS  
825 date to 2015 <sup>[52, 132]</sup>. In the work of Santos et al. <sup>[52]</sup> the ABS composed of PEG 8000 and  
826 sodium polyacrylate (NaPA) 8000 with 5% (w/w) ILs was used in extraction of cytochrome  
827 c (Cyt c). Several ILs comprising distinct cations and anions, namely imidazolium-based  
828 and ammonium-based ILs, were selected. It was seen that Cyt c preferentially partitioned  
829 to the bottom, NaPA 8000-rich phase, and the EE% were in most cases higher than those

830 obtained by the addition of NaCl or Na<sub>2</sub>SO<sub>4</sub> and complete extraction of Cyt c was observed.  
831 The partition behavior of Cyt c was attributed to the electrostatic interactions between the  
832 negatively charged protein and NaPA 8000, which were further increased by additional Cyt  
833 c-ILs interactions <sup>[52]</sup>. In the same year, the partitioning of BSA, lysozyme and myoglobin  
834 within PEG 600-potassium phosphate buffer ABS with 2.5, 5 or 7% (w/w) of [C<sub>2</sub>mim]Cl  
835 and [C<sub>4</sub>mim]Cl was investigated <sup>[132]</sup>. For both ILs used, the K of the proteins increased  
836 with the increase of the IL concentration added to the system when compared to the ABS  
837 without IL. Moreover, higher K were obtained in the systems containing [C<sub>4</sub>mim]Cl than  
838 with [C<sub>2</sub>mim]Cl, accordingly to the amount of these ILs found in the PEG-rich phase <sup>[132]</sup>.  
839 The authors concluded that the IL decreases the hydrophobic nature of the PEG-rich and  
840 therefore enhances the extraction of proteins to this phase <sup>[132]</sup>. The idea of using ILs as  
841 adjuvants in PEG-salt ABS for protein extraction was revisited in the work of our group in  
842 2019, in which the myoglobin partitioning behavior was studied <sup>[62]</sup>. In our study, the ABS  
843 composed of PEG 3350 and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was used and the effect of several imidazolium-,  
844 pyridinium- and pyrrolidinium-based ILs was evaluated. It was shown that myoglobin EE%  
845 and K increased with the IL hydrogen bond basicity ( $\beta$ ) and thus, the IL with highest  
846 hydrogen bond accepting character, [C<sub>4</sub>mim][CH<sub>3</sub>CO<sub>2</sub>] changed the myoglobin partitioning  
847 preferences from salt-rich to the PEG-rich phase <sup>[62]</sup>. Moreover, the increase of  
848 concentration of this IL from 5 to only 7.5% (w/w) allowed to obtain 100% EE% in the  
849 PEG-rich phase. Overall, in this work we showed that by appropriate choice of IL it is  
850 possible to tune properties of the PEG phase and use PEG-salt-IL-H<sub>2</sub>O quaternary ABS in  
851 either forward or back-extraction of myoglobin. In the most recent work, ILs were used as  
852 adjuvants in polymer-polymer ABS and the extractability of BSA and Cyt c tested <sup>[124]</sup>. The  
853 authors showed that the addition of chloride-based ILs as adjuvants resulted in either an  
854 increase or a decrease of protein's K, meaning that it was possible to tailor the proteins



855 partitioning between the phases of PEG-dextran ABS <sup>[124]</sup>. The partitioning experiments  
856 showed that in all systems BSA preferentially partitioned to the dextran-rich phase (the  
857 most hydrophilic phase in the investigated systems) and that the addition of ILs always  
858 increased the protein partitioning to the PEG-rich phase <sup>[124]</sup>. Furthermore, it was concluded  
859 that BSA partitioning essentially depends on the IL content in each phase <sup>[124]</sup>. On the other  
860 hand, Cyt c showed a K close to unity, meaning that there was no preference to any of the  
861 phases. However, the addition of 5% (w/w) of [C<sub>4</sub>mim]Cl, [C<sub>4</sub>mpyr]Cl, [P<sub>4444</sub>]Cl, and  
862 [N<sub>4444</sub>]Cl increased K values thus Cyt c was more prone to migrate to the PEG-rich phase  
863 <sup>[124]</sup>. Overall, partitioning of Cyt c was ruled by the ILs chemical structure and established  
864 interactions <sup>[124]</sup>.

865 The applicability of DES-based ABS in protein extraction was studied for the first time in  
866 the pioneering work regarding the use of DES as phase forming compounds of ABS <sup>[79]</sup>.  
867 The authors evaluated the performance of four DES (ChCl:urea (1:2), [N<sub>1111</sub>]Cl:urea (1:2),  
868 [N<sub>3333</sub>]Br:urea (1:2) and ChCl:methylurea (1:2)) with K<sub>2</sub>HPO<sub>4</sub> to yield ABS and these  
869 systems were used in BSA extraction <sup>[79]</sup>. In all these systems, the protein preferentially  
870 partitioned to the top, enriched in DES components, phase. The obtained EE% ranged from  
871 26.92 to 99.94% for [N<sub>3333</sub>]Br:urea (1:2) and ChCl:urea (1:2), respectively. These results  
872 indicate that the nature of the HBA in the DES have a significant impact on the BSA  
873 extraction. Furthermore, comparing the results obtained for the ChCl:urea and  
874 ChCl:methylurea (EE% of 34.39%) it can be also concluded that the influence of HBD  
875 cannot be discarded. The authors further used the ChCl:urea-based ABS in the extraction  
876 optimization procedure and the 100% EE% was reached <sup>[79]</sup>. It was stated that hydrophobic  
877 interactions, hydrogen bonding interactions and the salting-out effect played important roles  
878 in the BSA partitioning <sup>[79]</sup>. Later, Li et al. <sup>[112]</sup> prepared six DES using betaine as HBA,  
879 different HBDs (e.g. urea, methylurea, glucose, glycerol, sorbitol, ethylene glycol) and



880 water and combined them with  $K_2HPO_4$  to form ABS. The developed systems were then  
881 applied in extraction of BSA and ovalbumin. The obtained results showed that DES formed  
882 with different HBDs have distinct capabilities for extraction of these proteins. Much higher  
883 EE% were achieved for BSA than ovalbumin, in DES enriched phase <sup>[112]</sup>. The best system  
884 in extraction of BSA was composed with betaine:urea:H<sub>2</sub>O (1:2:1) DES, achieving 93.95%  
885 efficiency and 99.82% after optimization <sup>[112]</sup>. On the other hand, for ovalbumin, the highest  
886 EE% of only around 60% was obtained with betaine:ethylene glycol:H<sub>2</sub>O (1:2:1) DES <sup>[112]</sup>.  
887 In another report, ChCl:PEG 2000 DES was used to form ABS with three different salts:  
888  $NaH_2PO_4$ ,  $Na_2CO_3$ ,  $Na_3C_6H_5O_7$ , and these systems were applied in BSA partitioning <sup>[114]</sup>.  
889 The authors showed that the EE% decreased, following the order of phase forming ability  
890 of the salt:  $Na_2CO_3 > Na_3C_6H_5O_7 > NaH_2PO_4$ . Furthermore, it was shown that by changing  
891 the ratio of ChCl and PEG 2000 it is possible to improve the EE% from 54.42% to 83.50%  
892 for (1:1) and (20:1) molar ratios, respectively <sup>[114]</sup>. Overall, using the systems composed of  
893 ChCl:PEG (20:1) and  $Na_2CO_3$ , a BSA EE% of 95.16% was obtained, after optimization of  
894 extraction conditions (protein concentration, temperature, DES and salt amount, and pH of  
895 the system) <sup>[114]</sup>. Furthermore, DES- $K_2HPO_4$  ABS composed of binary ( $[N_{1111}]Cl$  as HBA  
896 and urea, glycerol, ethylene glycol, glucose as HBDs) and ternary DES ( $[N_{1111}]Cl$  as HBA,  
897 glycerol as HBD combined with different HBDs as urea, ethylene glycol, glucose, sorbitol)  
898 were used in the extraction of BSA, lysozyme and Cyt c <sup>[133]</sup>. The authors showed that the  
899 ternary DES have better extraction capability for the studied proteins than the binary DES.  
900 For instance, in the system composed of  $[N_{1111}]Cl$ :glycerol (1:2) DES, lower EE% were  
901 obtained than for the four ternary DES, in which glycerol was one of the two HBDs <sup>[133]</sup>.  
902 Moreover,  $[N_{1111}]Cl$ :glycerol:ethylene glycol (1:1:1) and  $[N_{1111}]Cl$ :glycerol:glucose (2:2:1)  
903 ternary DES were more advantageous for the extraction of studied proteins than  
904  $[N_{1111}]Cl$ :ethylene glycol (1:2) and  $[N_{1111}]Cl$ :glucose (1:1) <sup>[133]</sup>. The ABS formed with



905 [N<sub>1111</sub>]Cl:urea (1:2) and [N<sub>1111</sub>]Cl:glycerol:urea (1:1:1) were further investigated in BSA  
906 extraction optimization procedure and EE% in DES enriched phase of up to 99.31% and  
907 98.95% were obtained, respectively <sup>[133]</sup>. In another study, four ChCl-based DES, namely  
908 ChCl:ethylene glycol (1:2), ChCl:glycerol (1:1), ChCl:glucose (2:1), ChCl:sorbitol (1:1)  
909 were used to prepare ABS with K<sub>2</sub>HPO<sub>4</sub> and the extraction properties of these systems were  
910 evaluated using BSA as a model protein <sup>[113]</sup>. It was reported that ChCl:glycerol DES was  
911 the most suitable extraction solvent and after optimization procedure (the amount of DES,  
912 the concentration of salt, the mass of protein, the shaking time, the temperature and pH  
913 value) 98.16% BSA EE% was obtained in DES enrich phase in a single-step extraction <sup>[113]</sup>.  
914 Furthermore, it was showed that BSA kept its conformation after the extraction process and  
915 that the formation of DES–protein aggregates played a crucial role in the extraction  
916 mechanism <sup>[113]</sup>. Furthermore, the extraction potential of ABS formed with  
917 [N<sub>4444</sub>]Br:glycolic acid (1:1) DES and Na<sub>2</sub>SO<sub>4</sub> was studied using lysozyme as a model  
918 protein <sup>[134]</sup>. It was found out that more than 98% of protein was extracted to the DES  
919 enriched phase at the optimal conditions <sup>[134]</sup>. Moreover, the biological activity studies  
920 revealed that after the extraction lysozyme still retained 91.73% of initial activity <sup>[134]</sup>. In  
921 general, all these works showed that DES-based ABS have a great potential in extraction of  
922 different proteins and by using them we can take advantage of their tunability and prepare  
923 green, task-specific extractants with the desirable physicochemical properties.

### 924 **3.5. Virus and virus-like particles**

925 Virus and virus-like particles (VLPs) are biological therapeutic molecules used in various  
926 medical, analytical and scientific applications <sup>[135]</sup>. In particular, a major growth of interest  
927 in viral particles use in biomedical applications, such as vaccination, cancer therapy or as  
928 delivery vectors for gene therapy, has been observed <sup>[135]</sup>. For their use as biomedical agents,  
929 highly efficient and effective production (upstream process) and purification (downstream



930 process) process that will meet all regulatory requirements are essential. In the last decades,  
931 the upstream processes of virus and VLPs have been optimized and high yields and harvest  
932 volumes are currently obtained <sup>[135]</sup>. On the other hand, the downstream processing step,  
933 where the recovery and purification of the virus or VLPs is achieved, still constitutes the  
934 major drawback in overall productivity and cost of the manufacturing process <sup>[135]</sup>. The  
935 downstream processing of VLPs usually consists of different unit operations, essentially  
936 due to the complex structure and properties of these bioparticles and also to wide range of  
937 impurities generated in the upstream process <sup>[135]</sup>. The standard purification process of virus  
938 and VLPs is composed of several steps, which include (i) clarification with the removal of  
939 producer cells, cell debris and large aggregates by low speed centrifugation or  
940 microfiltration; (ii) concentration by centrifugation, ultrafiltration or precipitation and  
941 flocculation; (iii) purification and polishing by density gradient ultrafiltration or  
942 chromatography <sup>[135]</sup>. However, all these methods do not satisfy the economic requirements  
943 because they are time consuming, yield low product recovery, and are very difficult to scale-  
944 up <sup>[136]</sup>. For example, density gradient ultracentrifugation provides low yields, some  
945 impurities are still retained, and the implementation of the process is very difficult due to  
946 problems with scaling up <sup>[137-138]</sup>. The precipitation techniques possess low selectivity  
947 toward viral particles <sup>[139]</sup>, and in ultra- or microfiltration membrane clogging occurs and,  
948 thus, large impurities are also often retained and co-concentrated <sup>[140]</sup>. Consequently, to  
949 reduce these constraints, ABS have been successfully employed in the recovery of virus and  
950 VLPs as a promising alternative technique able to operate in a continuous mode.

951 A summary of results found in literature for the extraction and purification of VLPs using  
952 quaternary ABS are presented in Table 6.

953 The addition of neutral salts to ABS composed of PEG and salt for purification of  
954 recombinant VLPs from yeast cells homogenate was first evaluated in 1995 by Andrews et

955 al. <sup>[141]</sup>. In this work, cell debris removal was achieved using ABS composed of PEG 400  
956 or 600 and  $(\text{NH}_4)_2\text{SO}_4$ , while the separation of VLPs from contaminant proteins was  
957 obtained employing PEG 4000 or 8000 and  $(\text{NH}_4)_2\text{SO}_4$  ABS with NaCl or phosphate as  
958 additive. The authors showed that the addition of the salts had a significant impact on the  
959 effectiveness of these systems <sup>[141]</sup>. In another report, parvovirus B19 VLPs were  
960 successfully recovered from a clarified cell disruptate by interfacial partition using ABS  
961 composed of PEG 1000, magnesium sulfate and 800 mM NaCl as additive <sup>[142]</sup>. It was  
962 shown that majority of B19 VLPs preferentially partitioned to the interface, while the non-  
963 assembled VP2 proteins and host cell proteins migrated to either top or bottom phase. In  
964 general, 95.3% and 33.2% recovery yields of VP1 and VP2 proteins were obtained,  
965 respectively <sup>[142]</sup>. Purification of human B19 parvo-VLPs derived from *Spodoptera*  
966 *frugiperda Sf9* insect cells, using single- or multi-stage ABS extraction have been also  
967 reported <sup>[143]</sup>. The addition of NaCl to the PEG 400-phosphate buffer pH 8.5 ABS resulted  
968 in increased interfacial partitioning and precipitation of the VP2-VLPs. However, only  
969 about 20% of the viral particles were affected, while the rest VLPs were recovered in the  
970 top phase. Remarkably, a significant change in partitioning behavior of DNA from top  
971 phase to bottom phase was observed upon addition of NaCl <sup>[143]</sup>. Furthermore, the addition  
972 of NaCl slightly decreased the amount of contaminant proteins in the top phase. Overall,  
973 with addition of 7.5% (w/w) NaCl, a removal of up to 99% of DNA was achieved in one  
974 step and high selectivities were obtained for both single- and multi-stage ABS extraction  
975 <sup>[143]</sup>. Furthermore, Jacinto et al. <sup>[18]</sup> evaluated how addition of different concentrations of  
976 NaCl affected K of Human Immunodeficiency Virus (HIV) VLPs from CHO supernatants  
977 in PEG-dextran and PEG-salt ABS. This was the first report in which enveloped VLPs were  
978 purified using ABS. However, no significant improvements of K of HIV-VLPs were  
979 observed with the addition of NaCl <sup>[18]</sup>.





980 Osmolytes were also used as additives to enhance the purification of both virus (porcine  
981 parvovirus, PPV) and VLPs (HIV-VLPs) in a work of Heldt's group <sup>[47]</sup>. The authors used  
982 ABS composed of PEG with an average molecular weight of 12,000 Da and citrate buffer  
983 and added 0.5M of glycine, betaine, TMAO or urea <sup>[47]</sup>. It was reported that both glycine  
984 and betaine were able to increase the salting-out ability of the citrate-rich phase and the  
985 hydrophobicity of the PEG-rich phase, resulting in improved partitioning to the PEG-rich  
986 phase of PPV and HIV-VLPs at conditions, where the systems without osmolytes were  
987 insufficient to induce preferential partitioning <sup>[47]</sup>. Furthermore, it was observed that TMAO  
988 and urea were not capable to improve the virus and VLPs recovery <sup>[47]</sup>. Overall, recoveries  
989 of 100% for infectious PPV and 92% for the HIV-VLPs, with high removal of the  
990 contaminant proteins and more than 60% DNA removal when glycine was added were  
991 obtained <sup>[47]</sup>. Consequently, the following order of osmolytes efficiency to improve virus  
992 and VLPs purification was deducted: glycine > betaine > TMAO > urea <sup>[47]</sup>. The authors  
993 concluded that high recovery and purity of viral modalities in the PEG-rich phase in the  
994 presence of osmolytes was a result of the higher interfacial interactions for comparatively  
995 hydrophobic and rigid viruses compared to the intramolecular interactions of flexible  
996 proteins <sup>[47]</sup>.

997 ILs as adjuvants to polymer-salts ABS were used in our recent work, where an initial high-  
998 throughput screening was performed to find the most promising PEG-salt ABS for  
999 extraction of Hepatitis C Virus (HCV) VLPs <sup>[63]</sup>. After the screening and the optimization  
1000 of the extraction conditions, the ABS composed of PEG 400 and citrate buffer at pH 7 was  
1001 chosen and the effect of ILs on the extraction of HCV-VLPs was studied. We have selected  
1002 different imidazolium- and quaternary ammonium-based ILs, allowing to evaluate effect of  
1003 IL anion and the effect of the alkyl side chain length in the IL cation <sup>[63]</sup>. The extraction  
1004 studies revealed that the addition of 5% (w/w) [C<sub>4</sub>mim]Cl increased EE% from 87.9% (no



1005 IL) to 91.1%. Moreover, with addition of [C<sub>4</sub>mim][CH<sub>3</sub>CO<sub>2</sub>], the IL with a highest  
1006 hydrogen bonding basicity and ability to accept protons, higher extraction efficiency  
1007 (EE<sub>VLPs</sub>% = 90.3%) was obtained and VLPs recovery yield enhanced from 84.9% (system  
1008 without IL) to 88.5%. Furthermore, the advantageous effect of ILs was also confirmed in  
1009 the VLPs purification directly from clarified cell culture supernatants. The obtained results  
1010 showed that 100% VLPs EE% was attained in a single step. Moreover, with the addition of  
1011 [C<sub>4</sub>mim]Cl, the VLPs purity in the top phase was enhanced by 37% compared to the same  
1012 system without IL <sup>[63]</sup>. These promising results show that ILs can be very effective in  
1013 modulating the phase properties of polymer-salt ABS, achieving high HCV-VLP  
1014 purification, without hampering their structural and functional properties.

1015 In our following work, we extended the knowledge on the use of quaternary ABS in  
1016 extraction and purification of VLPs, using ABS composed of carbohydrates-based natural  
1017 deep eutectic solvents (NADES) <sup>[80]</sup>. We showed that HCV-VLPs have high affinity to the  
1018 carbohydrate/NADES enriched phase in most of the studied ABS and that the NADES-  
1019 based ABS are capable to enhance EE% of VLPs to the bottom phase compared to the  
1020 systems composed of each one of NADES components alone. The most promising ABS in  
1021 VLPs extraction was formed using fructose:sucrose (1:1) NADES and EE% of 99.6% were  
1022 obtained <sup>[80]</sup>. Furthermore, the potential of these systems in separation of VLPs from BSA  
1023 (the main contaminant protein in production of VLPs) was also evaluated. The obtained  
1024 results showed that BSA preferentially partitioned to the carbohydrate/NADES-rich phase  
1025 and fructose:glucose (1:1) NADES-based ABS extracted BSA with 99.5% efficiency <sup>[80]</sup>.  
1026 This system was shown to be the best suited to separate VLPs from BSA contaminants and  
1027 selectivity of 46.5 was attained, highlighting the potential of NADES-based ABS in VLPs  
1028 purification. Overall, these results highlight that by proper selection of NADES



1029 components, it is possible to finely tune the extractability and the separation efficiency of  
1030 ABS for the distinct biomolecules and achieve better selectivity of ABS.

#### 1031 **4. Biomolecules recovery, recycling and reusing of phase forming compounds**

1032 Having in mind industrial application of ABS for downstream processing and aiming at  
1033 further decreasing their environmental and economic impact, it is inevitable to advance  
1034 strategies for target biomolecule recovery and then recycling and reusing of phase forming  
1035 compounds. Achieving this is very often difficult and complex and it can compromise the  
1036 advantages of ABS in extraction and purification of biomolecules, such as their simplicity,  
1037 scalability, and high efficiency. Furthermore, the addition of unit operations for the removal  
1038 of phase forming chemicals from the product of interest also increases costs and complexity  
1039 of the process. Moreover, it must be noted that in quaternary ABS, the presence of fourth  
1040 compound in biphasic mixture turned this task even more complex as sometimes more unit  
1041 operations are required to achieve this task.

1042 Over the years, different strategies have been studied to conveniently recover target  
1043 biomolecule from phase forming compounds. Usually, these strategies take advantage of  
1044 size or chemical differences between the target biomolecule and the ABS phase forming  
1045 chemicals and they include ultrafiltration, diafiltration, dialysis or precipitation. Moreover,  
1046 other strategies such as back-extraction or induced phase separation (temperature) were also  
1047 studied.

1048 Ultrafiltration/diafiltration (UF/DF) has been used to recover target biomolecule from the  
.049 top <sup>[144-146]</sup> or bottom <sup>[147-150]</sup> phase of different ABS. This method enables to separate the  
.050 target biomolecule from phase forming chemicals by size with the use of a porous  
.051 membrane. Moreover, UF/DF can be used both in laboratorial and large scale and it also  
.052 allows to further concentrate the final product by reduction of the volume. Another approach

1053 for the recovery of target biomolecule described in the literature is dialysis. For instance, a  
1054 successful attempt of BSA recovery from IL-rich phase was described in the work of Pereira  
1055 et. al <sup>[151]</sup>. The authors showed that after protein removal by dialysis, IL can be reused for  
1056 two more extraction cycles without decreasing their high EE% for BSA (ca. 100%) <sup>[151]</sup>. In  
1057 another work, dialysis was also used to recover lipase from *Bacillus* sp. ITP-001 and  
1058 contaminant proteins from the polymer- and salt-rich phases of PEG-salt-IL quaternary  
1059 ABS, respectively <sup>[67]</sup>. The authors showed that after the dialysis of each phase, the enzyme  
1060 and contaminant proteins can be successfully recovered, and each phase can be reused for  
1061 new extraction and purification cycles <sup>[67]</sup>. Another strategy very often used in order to  
1062 separate proteins from phase forming compounds is precipitation <sup>[152]</sup>. In particular, in the  
1063 case of ABS affinity precipitation is used in which the target molecule is connected to a  
1064 specific ligand added to the system (without being a phase forming chemical) and after the  
1065 separation of the phases, the complex of target molecule and ligand is precipitated <sup>[153-155]</sup>.

1066 Another alternative strategy is back-extraction. In this approach a top phase of a system  
1067 where the biomolecules partitioned in a first system is transferred to a second fresh bottom,  
1068 salt-rich phase with a different composition. In that way, the target biomolecule partitions  
1069 into the bottom phase of this second system, thus obtaining top phase without target  
1070 biomolecule that would be reutilized in another extraction cycle <sup>[17, 122, 156]</sup>. Furthermore, in  
1071 our work we showed that by using ILs as adjuvants to PEG-salt ABS it is possible to  
1072 manipulate protein affinity to the phases by using adequate IL and use such systems for  
1073 extraction and back-extraction, making such quaternary ABS a very versatile extraction  
1074 platform <sup>[62]</sup>.

.075 Furthermore, thermosensitive polymers were also used in ABS formation. This property of  
.076 polymers allowed their recover and reuse in the next extraction cycles by increasing the  
.077 temperature of the system. For example, in the work of Li et. al, the PPG recycling process



1078 was proposed where the increase in the temperature drives PPG from the aqueous solution  
1079 of cholinium propionate + PPG 400, allowing the concentration of PPG 400 in the polymer-  
1080 rich phase to circa 90 % (w/w) at 45°C, achieving the recyclability of 90% of PPG 400 [157].  
1081 In another work, ABS composed of thermo-sensitive ethylene oxide–propylene oxide  
1082 (EOPO) random copolymer and salt was used to extract polyphenols from *Camellia sinensis*  
1083 *var. assamica* leaves [158]. After the extraction process the authors proposed the EOPO  
1084 polymer recovery by thermo-induction of the polymer-rich phase, allowing to recover  
1085 95.2% of the EOPO 2500 polymer [158].

1086 However, DES-based quaternary ABS very often do not require the recovery of target  
1087 biomolecule from ABS phase forming chemicals. Due to the high number of DES starting  
1088 materials that are non-toxic, biocompatible, and used as excipients in formulation of the  
1089 final bioproduct, it is possible directly use the phase to which biomolecule partitioned. The  
1090 examples of such ABS were shown in our works where the extraction and purification of  
1091 VLPs [80] and pepsin [115] was studied using DES-based ABS. Furthermore, since the  
1092 polymer used in ABS formation was PPG, it also opens a possibility of recycling of the  
1093 PPG-rich phase by thermo-induction.

## 1094 **5. Conclusions and outlook**

1095 Aqueous biphasic systems extraction has been recognized as an attractive and alternative  
1096 methodology in downstream processing of various biomolecules, mainly because of its  
1097 simplicity, selectivity, high capacity, and easy scalability. However, even though much  
1098 effort has been put into their development and a lot of advantages associated with the use  
1099 of ABS have been acknowledged, no major advances in introducing this methodology in  
1100 industries have been made in the last decades. The reason behind this is, primarily, the lack  
1101 of predictive models due to the poor overall understanding of the partition mechanisms

1102 involved in ABS. Furthermore, the implementation of ABS in large scale may raise some  
1103 environmental, safety and economic concerns due to the great amount of chemicals (e.g.  
1104 salts and polymers, ILs) needed to form ABS, further resulting in increased costs of the  
1105 process. Moreover, the most of biomolecules already have their purification process well  
1106 established and to replace it by ABS it would be necessary to change the existing  
1107 infrastructures. In our opinion ABS have higher opportunity to be introduced in industrial  
1108 downstream processes of newly developed biomolecules which have not well-defined  
1109 purification process, or for the biomolecules that are currently purified using expensive and  
1110 time-consuming process. We believe that, perhaps, the reluctance of the industry may be  
1111 overcome by introduction of some modification to the conventional ABS. In particular, as  
1112 discussed in this review, quaternary ABS formed with the addition of different chemicals,  
1113 such as neutral salts, ILs or those composed of DES, can lead to a powerful technology to  
1114 purify biomolecules with high quality, while being less expensive and safer to the  
1115 environment than the conventional systems and thus being the potential answer on the  
1116 doubts raised by the industry. Throughout this review, we show the advantages of  
1117 quaternary systems over conventional ABS. For example, the addition of NaCl to polymer-  
1118 salt was shown to be very effective in enhancing extraction and purification of monoclonal  
1119 antibodies. Furthermore, the use of osmolytes as additives to polymer-salt ABS or DES  
1120 composed of osmolytes combined with polymer to yield ABS seems to be an interesting  
1121 approach to increase the purification of virus and VLPs, while enhancing their stability due  
1122 to common use of these substances as excipients in vaccine formulation. Overall, due to  
1123 high number of possible ILs, these chemicals offer the possibility to manipulate  
1124 extractability of very wide range of biomolecules such as amino acids, enzymes, proteins,  
1125 monoclonal antibodies, VLPs, among others. However, their use should be well-thought-  
1126 out due to the toxicity of some of them. Thus, envisaging extraction and purification of



1127 biomolecules relevant in industries, in which biocompatibility is required, DES should be  
1128 considered as viable alternative to ILs as they were shown as effective but greener than ILs.

1129 Beyond effectiveness of quaternary ABS in the extraction and purification of many different  
1130 types of biomolecules, these systems are able to decrease the amounts of the phase forming  
1131 compounds needed for the liquid-liquid demixing. Moreover, the possibility of using natural  
1132 components, either in the synthesis of ILs or preparation of DES, opened exciting new  
1133 perspectives to design truly sustainable and biocompatible solvents for the quaternary ABS  
1134 implementation. Undoubtedly, the new compounds used to create quaternary ABS will  
1135 result in further reduction of the costs. What is more, quaternary ABS formed using DES  
1136 offer the possibility of integrated bioprocess, where the recovery of the target biomolecule  
1137 can be avoided. In that way, even more competitive and sustainable downstream process  
1138 using ABS than those used nowadays can be developed.

1139 It is expected that, in a near future, the theoretical and experimental knowledge about  
1140 quaternary ABS will evolve rapidly and these systems will surely gain more importance.  
1141 Nevertheless, there are still many challenges before ABS could be implemented in industrial  
1142 processes. For instance, more attention should be paid to the development of large-scale  
1143 industrial settlements for ABS technology. Also, more studies on the design of integrated  
1144 and continuous bioprocesses using quaternary ABS should be performed. Furthermore,  
1145 predictive models for the behavior of ABS and biomolecules partition should still be  
1146 studied, allowing to estimate K or specific ABS conditions. Moreover, it is believed that  
1147 ABS will continue to have a significant research interest for biomolecules purification and  
1148 recovery. In that way, in the near future more effective, sustainable and robust ABS at  
1149 different scales will be developed, leading to commercial implementation of this technology  
1150 at large scale recovery of high-value biomolecules.



1151

1152 **Disclosure statement**

1153 The authors declare that they have no competing interests.

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1684 **Table 1:** Name and acronym of the compounds used in quaternary ABS implementation  
 1685 considered in this review.

Type	Name	Acronym
<b>Ionic liquids</b>	1-alkyl-3-methylimidazolium acetate	[C <sub>n</sub> mim][CH <sub>3</sub> CO <sub>2</sub> ]
	1-alkyl-3-methylimidazolium bromide	[C <sub>n</sub> mim]Br
	1-alkyl-3-methylimidazolium chloride	[C <sub>n</sub> mim]Cl
	1-alkyl-3-methylimidazolium dicyanide	[C <sub>n</sub> mim][N(CN) <sub>2</sub> ]
	1-alkyl-3-methylimidazolium dimethylphosphate	[C <sub>n</sub> mim][[(CH <sub>3</sub> ) <sub>2</sub> PO <sub>4</sub> ]
	1-alkyl-3-methylimidazolium methanesulfonate	[C <sub>n</sub> mim][CH <sub>3</sub> SO <sub>3</sub> ]
	1-alkyl-3-methylimidazolium tosylate	[C <sub>n</sub> mim][TOS]
	1-alkyl-3-methylimidazolium trifluoromethanesulfonate	[C <sub>n</sub> mim][CF <sub>3</sub> SO <sub>3</sub> ]
	1-benzyl-3-methylimidazolium chloride	[C <sub>7</sub> H <sub>7</sub> mim]Cl
	1-hydroxyalkyl-3-methylimidazolium chloride	[OHC <sub>n</sub> mim]Cl
	Tetrabutylammonium chloride	[N <sub>4444</sub> ]Cl
	Tetrabutylphosphonium chloride	[P <sub>4444</sub> ]Cl
	Tetramethylammonium chloride	[N <sub>1111</sub> ]Cl
Tetrapropylammonium bromide	[N <sub>3333</sub> ]Br	
<b>Osmolytes</b>	Amino acid	AA
	Betaine hydrochloride	BeHCl
	Guanidine hydrochloride	GuHCl
	Trimethylamine N-oxide	TMAO
<b>Polymers</b>	Polyacrylic acid	PAA
	Polyethylene glycol	PEG

	Polypropylene glycol	PPG
	Sodium polyacrylate	NaPA
<b>Salts</b>	Ammonium sulfate	$(\text{NH}_4)_2\text{SO}_4$
	Potassium chloride	KCl
	Sodium chloride	NaCl
	Sodium perchlorate	$\text{NaClO}_4$
	Choline chloride	ChCl

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1703 **Table 2:** Quaternary ABS and representative results reported in literature for the extraction  
 1704 and purification of AAs. EE% = extraction efficiency; K = partition coefficient; S =  
 1705 selectivity.

Amino acid	ABS composition	Main results	Reference
<b>L-tryptophan</b>	PEG 600 + Na <sub>2</sub> SO <sub>4</sub> + H <sub>2</sub> O + [C <sub>7</sub> H <sub>7</sub> mim]Cl	K - 20.54 (no IL) to 42.47 (with IL)	[27]
	PEG 400 + (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> + H <sub>2</sub> O + [N <sub>4444</sub> ]Cl	K - 7.4 (no IL) to 14.6 (with IL)	[64]
	PEG 400 + citrate buffer pH 7 + H <sub>2</sub> O + [N <sub>4444</sub> ]Cl	K - 13.48 (no IL) to 24.39 (with IL)	[66]
	ChCl:glucose (1:1) + PPG 400 + H <sub>2</sub> O	EE% - 90.59% in ChCl enriched phase	[28]
	ChCl:1,2-propanodiol (1:1) + K <sub>2</sub> HPO <sub>4</sub> + H <sub>2</sub> O	EE% - 98.7% in ChCl enriched phase S <sub>Trp/Tyr</sub> - 30.9	[30]
	ChCl:PEG 2000 (2:3) + K <sub>2</sub> HPO <sub>4</sub> + H <sub>2</sub> O	EE% - 93.88% in DES enriched phase	[101]
<b>L-tyrosine</b>	PEG 400 + (NH <sub>4</sub> )SO <sub>4</sub> + H <sub>2</sub> O + [P <sub>4444</sub> ]Cl	K - 2.3 (no IL) to 3.4 (with IL)	[64]
	PEG 400 + citrate buffer pH 7 + H <sub>2</sub> O + [P <sub>4444</sub> ]Cl	K - 4.85 (no IL) to 7.23 (with IL)	[66]
	ChCl:glucose (1:1) + PPG 400 + H <sub>2</sub> O	EE% - 83.55% in DES enriched phase	[28]
	ChCl:ethanol (2:1) + K <sub>2</sub> HPO <sub>4</sub> + H <sub>2</sub> O	EE% - 86.7% in ChCl enriched phase	[30]
<b>L-phenylalanine</b>	PEG 400 + citrate buffer pH 7 + H <sub>2</sub> O + [N <sub>4444</sub> ]Cl	K - 7.58 (no IL) to 13.39 (with IL)	[66]
	ChCl:glucose (1:1) + PPG 400 + H <sub>2</sub> O	EE% - 86.69% in DES enriched phase	[28]
	ChCl:ethanol (2:1) + K <sub>2</sub> HPO <sub>4</sub> + H <sub>2</sub> O	EE% - 94.3% in ChCl enriched phase	[30]

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1709 **Table 3:** Quaternary ABS and representative results reported in literature for the extraction  
 1710 and purification of enzymes. EE% = extraction efficiency; K = partition coefficient.

Enzyme	ABS composition	Main results	Reference
<b>Lipase</b>	2-propanol + K <sub>2</sub> HPO <sub>4</sub> + H <sub>2</sub> O + 4.5% (w/v) NaCl	Recovery – 76% (no NaCl) to 99% (with NaCl) Purity - 6.4-fold (no NaCl) to 13.5-fold (with NaCl)	[107]
	PEG 8000 + potassium phosphate + H <sub>2</sub> O + 6% (w/w) NaCl	Purity - 59.93-fold (no NaCl) to 141.65-fold (with NaCl)	[108]
	PEG 1500 + K <sub>2</sub> HPO <sub>4</sub> /KH <sub>2</sub> PO <sub>4</sub> + H <sub>2</sub> O + [C <sub>6</sub> mim]Cl	Purity 175.61-fold (no IL) to 245-fold (with IL)	[67]
<b>Protease</b>	PEG 4000 + MgSO <sub>4</sub> + H <sub>2</sub> O + 6% (w/w) NaCl	Recovery - 23.58% (no NaCl) to 107% (with NaCl)	[109]
	PEG + dextran + H <sub>2</sub> O + 4.5% (w/w) NaCl	K - 84.2 Purity - 14.37-fold Yield - 97.3%	[111]
<b>Invertase</b>	PEG 3000 + Na <sub>2</sub> SO <sub>4</sub> + H <sub>2</sub> O + 5% (w/w) KCl	Recovery - 68% (no KCl) to 90% (with KCl) Purity - 3.3-fold (no KCl) to 5.5-fold (with KCl)	[110]
<b>L-asparaginase</b>	PEG 6000 + citrate buffer + H <sub>2</sub> O + [C <sub>4</sub> mim][CH <sub>3</sub> SO <sub>3</sub> ]	Recovery - 87.94% Purity – 20.09-fold Specific activity -3.61 U/mg	[65]
<b>Trypsin</b>	ChCl:urea (1:2) + K <sub>2</sub> HPO <sub>4</sub> + H <sub>2</sub> O	EE% - 95.53% in DES enriched phase	[79]
	Betaine:methylurea (1:2) + K <sub>2</sub> HPO <sub>4</sub> + H <sub>2</sub> O	EE% - >90% in DES enriched phase	[112]
	ChCl:glycerol (1:1) + K <sub>2</sub> HPO <sub>4</sub> + H <sub>2</sub> O	EE% - 94.36% in DES enriched phase	[113]
<b>Papain</b>	ChCl:PEG 2000 (20:1) + Na <sub>2</sub> CO <sub>3</sub> + H <sub>2</sub> O	EE% - 90.95% in DES enriched phase	[114]
<b>Pepsin</b>	BeHCl:glucose (1:1) + PPG 425 + H <sub>2</sub> O	Activity recovery - 141.9% EE% - 99.5% in DES enriched phase	[115]

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1715 **Table 4:** Quaternary ABS and representative results reported in literature for the extraction  
 1716 and purification of monoclonal antibodies. EE% = extraction efficiency.

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Antibody	ABS composition	Main results	Reference
IgG	PEG 1450 + phosphate + H <sub>2</sub> O + 12% (w/w) NaCl	Purity - 80% Purification fold - 5.9-fold Recovery yield - 100%	[119]
	PEG 3350 + phosphate buffer pH 6 + H <sub>2</sub> O + 15% (w/w) NaCl	Recovery yield - 101% Purity - 99% Yield - 97%	[118]
	PEG 6000 + phosphate buffer pH 6 + H <sub>2</sub> O + 15% (w/w) NaCl	Recovery yield - 88% (CHO cells culture supernatants) and 90% (hybridoma cell culture supernatants) Purification factor - 4.3 (CHO cell culture supernatants) and 4.1 (hybridoma cell culture supernatants)	[117]
	PEG 3350 + phosphate buffer pH 6 + H <sub>2</sub> O + 10% (w/w) NaCl	Recovery - 89% Purity - 75%	[24]
	PEG 3350 + phosphate buffer pH 6 + H <sub>2</sub> O + NaCl	Recovery yield - 80% (CHO cell culture supernatants) and 100% (PER.C6® cell culture supernatants)	[122]
	PEG 400 + citrate buffer pH 7 + H <sub>2</sub> O + 5% (w/w) [C <sub>4</sub> mim][CH <sub>3</sub> CO <sub>2</sub> ]	EE% - 100% in PEG 400-rich phase Purity - 37% enhancement with IL	[61]
	PEG 6000 + dextran 450-650 + H <sub>2</sub> O + [C <sub>4</sub> mim]Cl, [C <sub>4</sub> mpyr]Cl, [C <sub>4</sub> mpip]Cl, [P <sub>4444</sub> ]Cl, [N <sub>4444</sub> ]Cl	K - ↑ with [C <sub>4</sub> mim]Cl, [C <sub>4</sub> mpyr]Cl, [N <sub>4444</sub> ]Cl, [C <sub>4</sub> mpip]Cl S <sub>Cyt c</sub> /IgG - ↑ with [C <sub>4</sub> mpyr]Cl, [N <sub>4444</sub> ]Cl, [P <sub>4444</sub> ]Cl than without IL S <sub>BSA</sub> /IgG - ↑ with [C <sub>4</sub> mim]Cl, [C <sub>4</sub> mpip]Cl than without IL	[124]

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1724 **Table 5:** Quaternary ABS and representative results reported in literature for the extraction  
 1725 and purification of proteins. EE% = extraction efficiency; K = partition coefficient.

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Protein	ABS composition	Main results	Reference
BSA	ChCl:urea (1:2) + K <sub>2</sub> HPO <sub>4</sub> + H <sub>2</sub> O	EE% - 99.94% in DES enriched phase	[79]
	Betaine:urea (1:2) + K <sub>2</sub> HPO <sub>4</sub> + H <sub>2</sub> O	EE% - 99.82% in DES enriched phase	[112]
	ChCl:glycerol (1:1) + K <sub>2</sub> HPO <sub>4</sub> + H <sub>2</sub> O	EE% - 98.16% in DES enriched phase	[113]
	ChCl:PEG 2000 (20:1) + Na <sub>2</sub> CO <sub>3</sub> + H <sub>2</sub> O	EE% - 95.16% in DES enriched phase	[114]
	[N <sub>1111</sub> ]Cl:urea (1:2) + K <sub>2</sub> HPO <sub>4</sub> + H <sub>2</sub> O	EE% - 99.31% in DES enriched phase	[133]
	PEG 6000 + dextran 450-650 + H <sub>2</sub> O + [C <sub>4</sub> mim]Cl, [C <sub>4</sub> mpyr]Cl, [C <sub>4</sub> mpip]Cl, [P <sub>4444</sub> ]Cl, [N <sub>4444</sub> ]Cl	K - ↑ with ↑ IL concentration	[124]
Cytochrome C	PEG 8000 + NaPA 8000 + H <sub>2</sub> O + [C <sub>2</sub> mim][(CH <sub>3</sub> ) <sub>2</sub> PO <sub>4</sub> ], [C <sub>2</sub> mim][CH <sub>3</sub> SO <sub>3</sub> ], [C <sub>2</sub> mim][CH <sub>3</sub> CO <sub>2</sub> ], [C <sub>2</sub> mim]Cl, [C <sub>2</sub> mim][CF <sub>3</sub> SO <sub>3</sub> ], [OHC <sub>2</sub> mim]Cl	EE% - 100% in the NaPA 8000-rich phase	[52]
	PEG 6000 + dextran 450-650 + H <sub>2</sub> O + [C <sub>4</sub> mim]Cl, [C <sub>4</sub> mpyr]Cl, [P <sub>4444</sub> ]Cl, [N <sub>4444</sub> ]Cl	K - ↑ (with IL)	[124]
Lysozyme	PEG 600 + phosphate buffer + H <sub>2</sub> O + [C <sub>2</sub> mim]Cl or [C <sub>4</sub> mim]Cl	K - ↑ with ↑ IL concentration	[132]
	[N <sub>4444</sub> ]Br:glycolic acid (1:1) + Na <sub>2</sub> SO <sub>4</sub> + H <sub>2</sub> O	EE% - >98% in DES enriched phase	[134]
Myoglobin	PEG 4000 + PAA + H <sub>2</sub> O + NaCl	K - 4.20 (no NaCl) to 15.77 (with NaCl)	[20]
	PEG 600 + phosphate buffer + H <sub>2</sub> O + [C <sub>2</sub> mim]Cl or [C <sub>4</sub> mim]Cl	K - ↑ with ↑ IL concentration	[132]
	PEG 3350 + NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> + H <sub>2</sub> O + 7.5% (w/w) [C <sub>4</sub> mim][CH <sub>3</sub> CO <sub>2</sub> ]	EE% - 100% in PEG 3350-rich phase	[62]
Ovalbumin	PEG 4000 + PAA + H <sub>2</sub> O + NaCl	K - 2.82 (no NaCl) to 5.51 (with NaCl)	[20]
	Betaine:ethylene glycol (1:2) + K <sub>2</sub> HPO <sub>4</sub> + H <sub>2</sub> O	EE% - ~60% in DES enriched phase	[112]

<b>Thaumatococcus</b>	PEG 6000 + phosphate + H <sub>2</sub> O + 1.5 M NaCl	K - ↑ (with NaCl) Recovery yield - 90-95% Purification - 20-fold	[127]
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1729 **Table 6:** Quaternary ABS and representative results reported in literature for the extraction  
1730 and purification of virus or virus-like particles. EE% = extraction efficiency; S = selectivity.

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<b>Virus or VLPs</b>	<b>ABS composition</b>	<b>Main results</b>	<b>Reference</b>
<b>Parvovirus B19 VLPs</b>	PEG 1000 + MgSO <sub>4</sub> + H <sub>2</sub> O + 800 mM NaCl	Recovery yield - 95.3% (VP1 protein) and 33.2% (VP2 protein)	[142]
	PEG 400 + phosphate buffer pH 8.5 + H <sub>2</sub> O + 7.5% (w/w) NaCl	DNA removal - 99% S - ↑ (with NaCl)	[143]
<b>Porcine parvovirus</b>	PEG 12000 Da + citrate buffer pH 7 + 0.5M glycine + H <sub>2</sub> O	Recovery yield - 100% DNA removal - >60%	[47]
<b>HCV-VLPs</b>	PEG 400 + citrate buffer pH 7 + H <sub>2</sub> O + [C <sub>4</sub> mim]Cl	EE% - 100% in PEG 400-rich phase Purity - 37% enhancement with IL	[63]
	Fructose:glucose (1:1) + PPG 425 + H <sub>2</sub> O	EE% - ↑ than for ternary ABS S <sub>VLPs/BSA</sub> - 46.5	[80]
<b>HIV-VLPs</b>	PEG 12000 Da + citrate buffer pH 7 + 0.5M glycine + H <sub>2</sub> O	Recovery yield - 92% DNA removal - >60%	[47]

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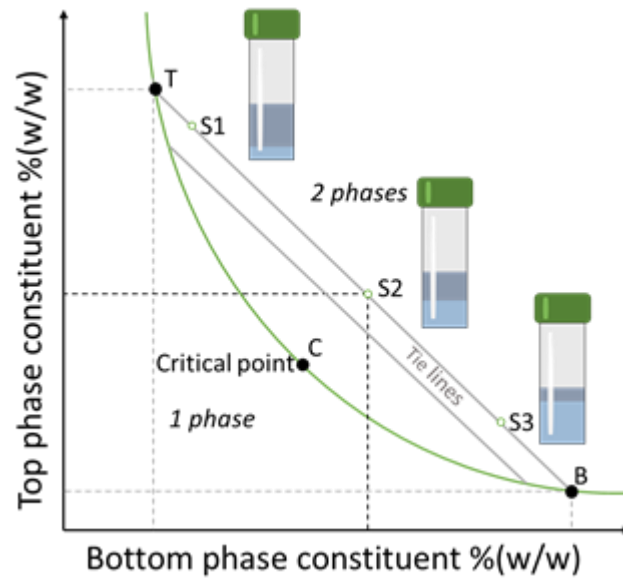
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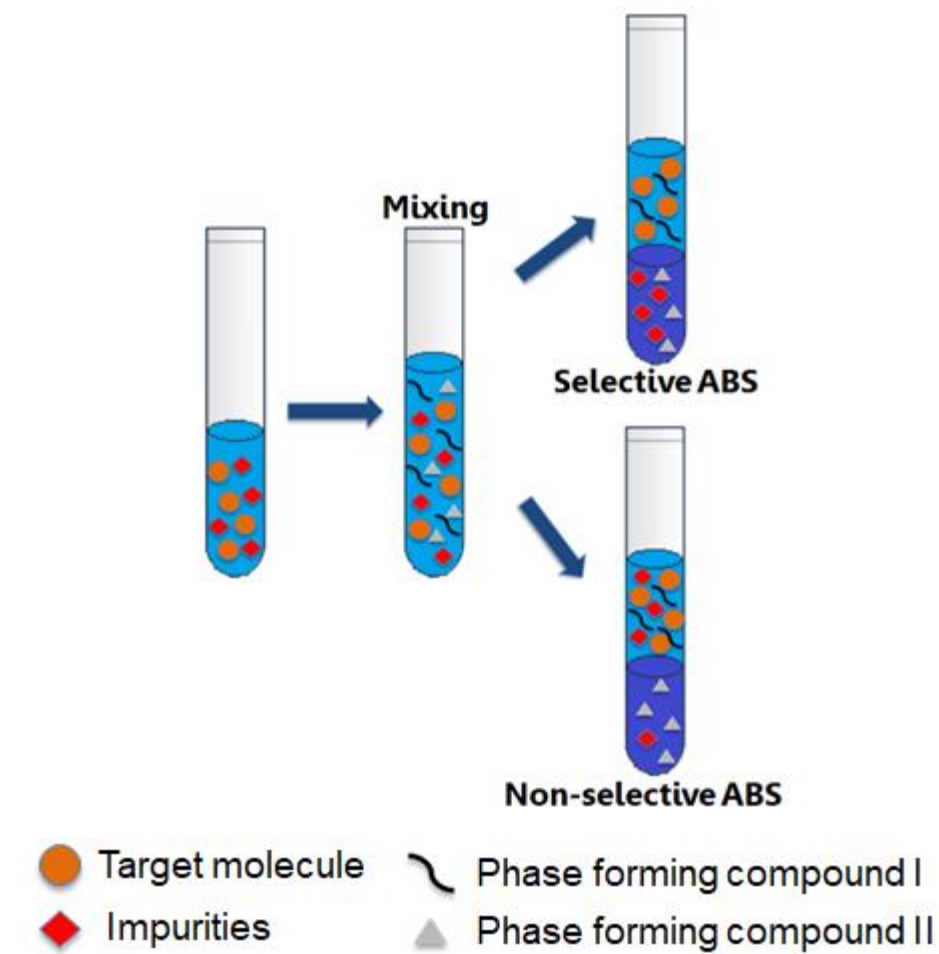
1745

1746 Fig. 1

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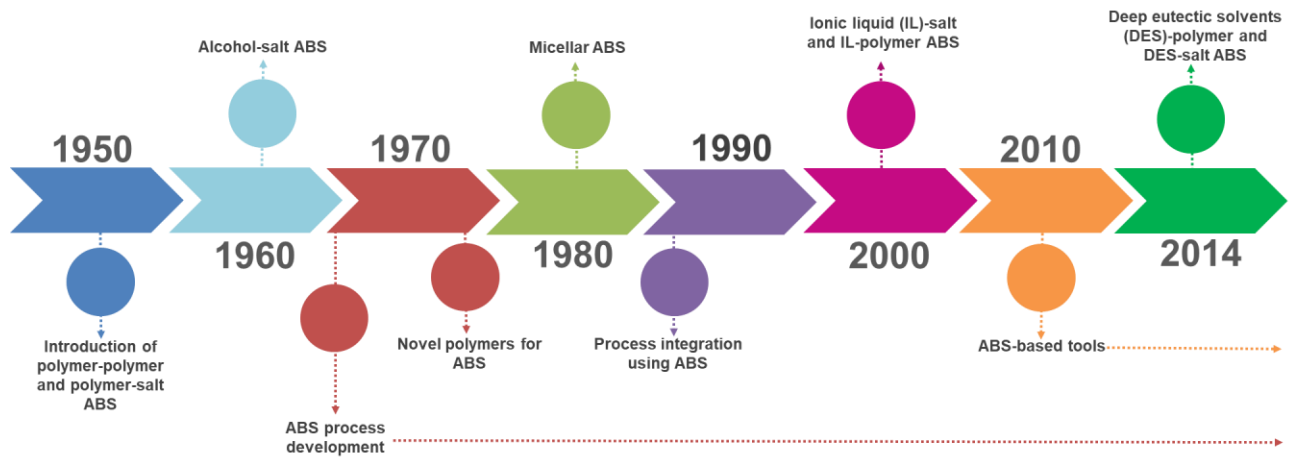
1751 Fig. 2

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1757 Fig. 3

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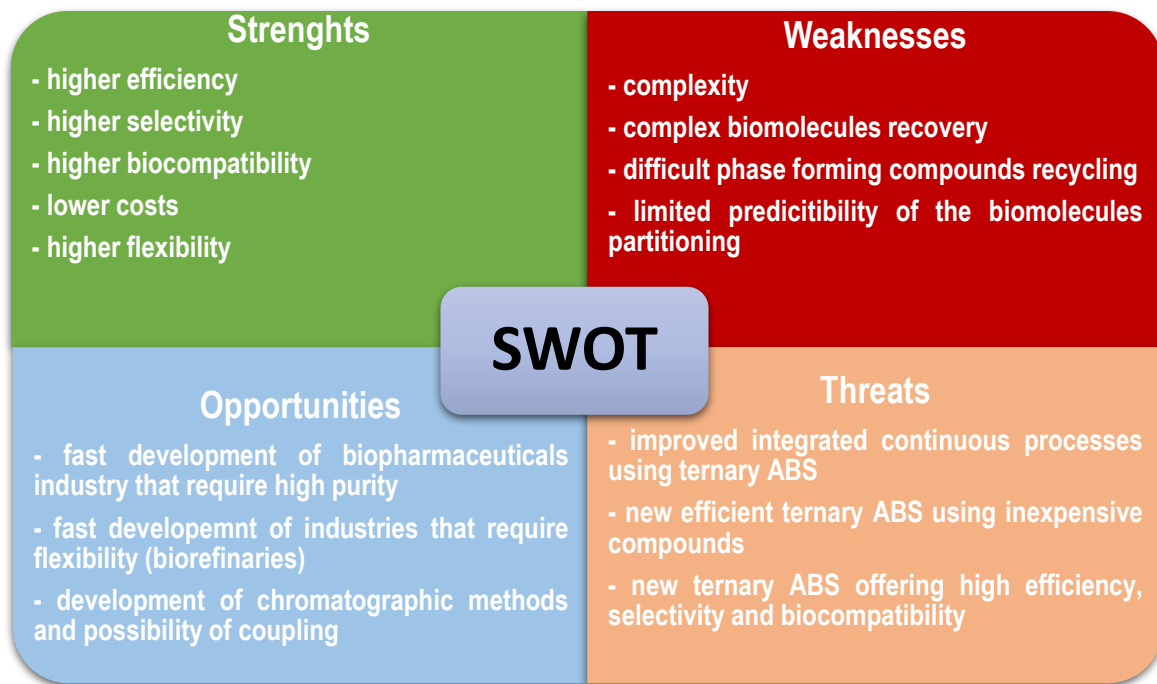
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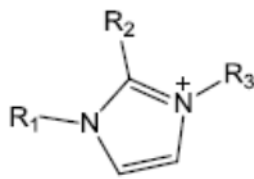
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1765 Fig. 4

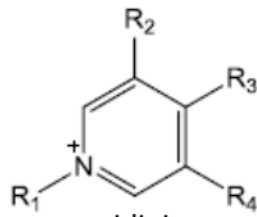
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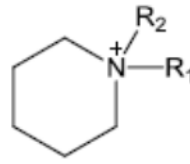
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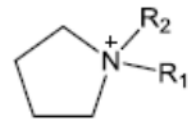
imidazolium



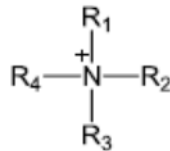
pyridinium



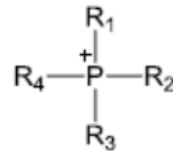
piperidinium



pyrrolidinium

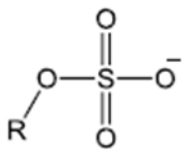


ammonium

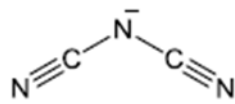


phosphonium

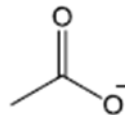
## Anions



alkylsulfate



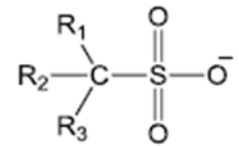
dicyanamide



acetate



halide



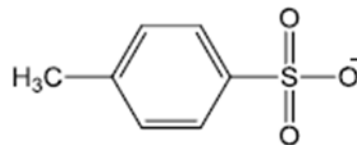
methanesulfonate



hexafluoro-  
phosphate



tetrafluoro-  
borate



tosylate

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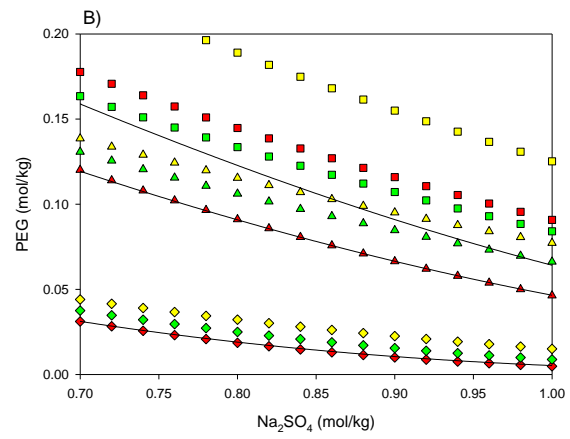
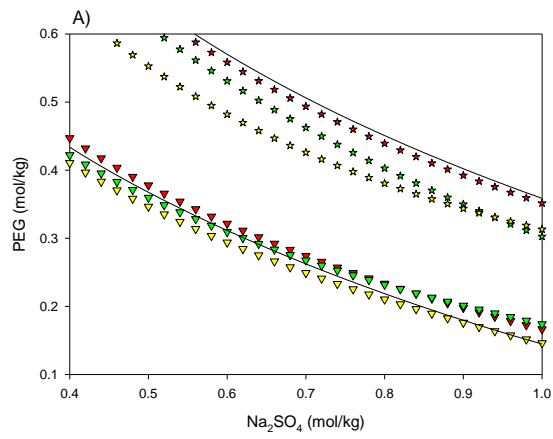
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Fig. 5

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Fig. 6

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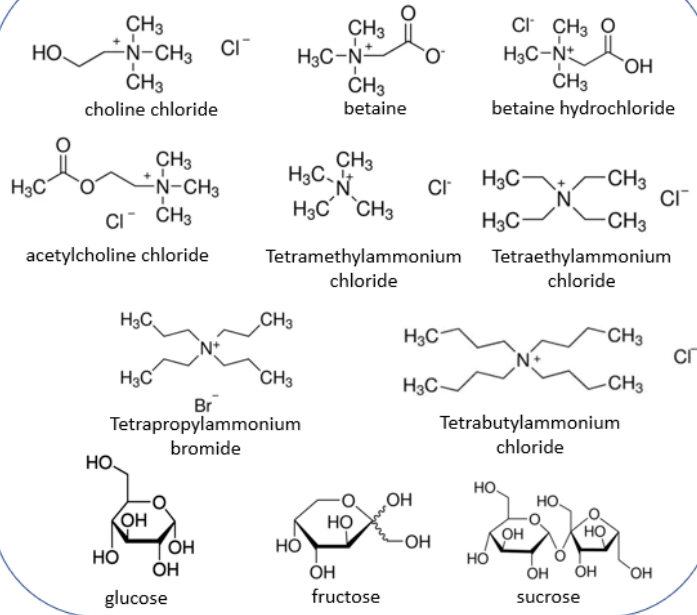
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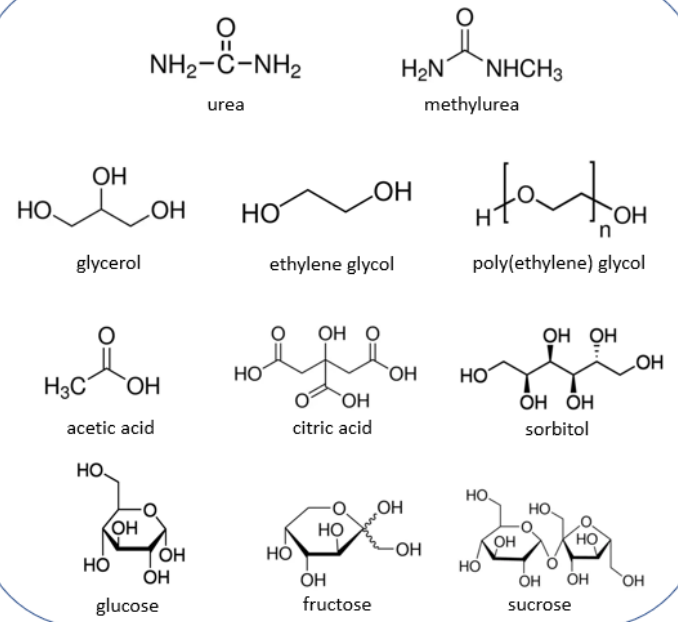
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### Hydrogen Bond Acceptors



### Hydrogen Bond Donors



1781 Fig. 7

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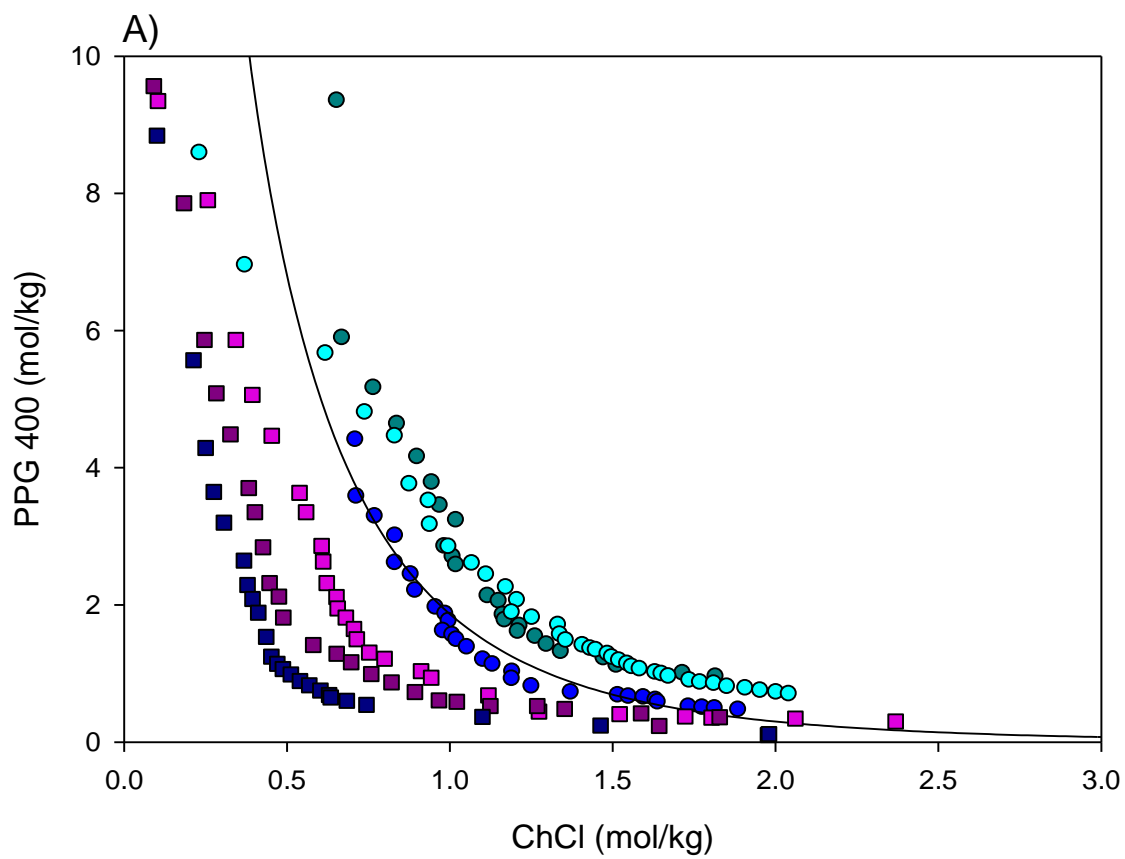
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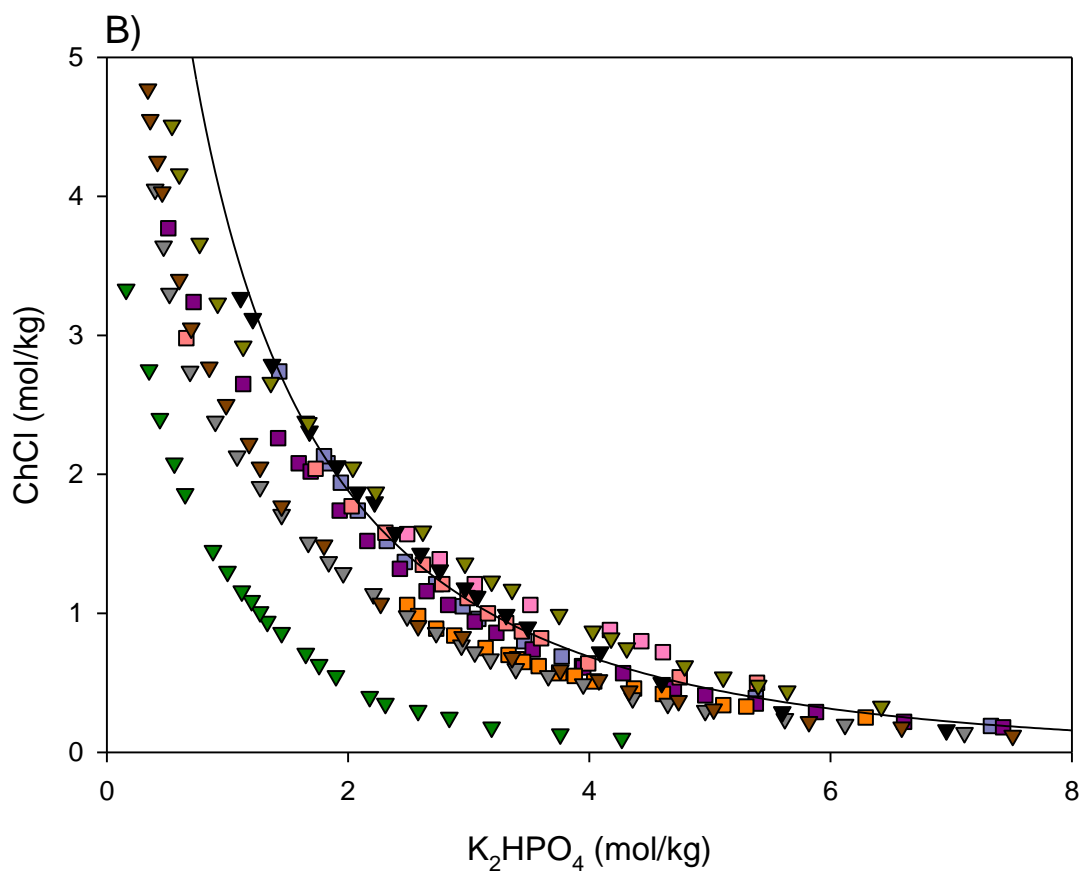
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.790

.791 Fig. 8

1792 **Fig. 1:** Schematic representation of a phase diagram for an aqueous biphasic system. T =  
1793 top phase composition; B = bottom phase composition; S1, S2, S3 = overall system  
1794 compositions; C = critical point.

1795 **Fig. 2:** Schematic diagram illustrating ABS formation and partitioning of target molecule  
1796 and contaminant proteins (impurities) in a ABS.

1797 **Fig. 3:** Important landmarks in aqueous biphasic systems (ABS) history.

1798 **Fig. 4:** Summary of the main strengths, weaknesses, opportunities, and threats (SWOT  
1799 analysis) of the quaternary ABS compared to the conventional ABS for the downstream  
1800 processing of biomolecules.

1801 **Fig. 5:** Examples of the chemical structures of cations and anions present in common ionic  
1802 liquids.

1803 **Fig. 6:** Binodal curves for the systems composed of: **A)** PEG 400 ( $\star$ ) or PEG 600 ( $\nabla$ ) +  
1804  $\text{Na}_2\text{SO}_4 + \text{H}_2\text{O} + 5\%$  (w/w) of  $[\text{C}_2\text{mim}]\text{Cl}$  (red),  $[\text{C}_4\text{mim}]\text{Cl}$  (green),  $[\text{C}_6\text{mim}]\text{Cl}$  (yellow);  
1805 and **B)** PEG 800 ( $\square$ ), PEG 1000 ( $\triangle$ ) or PEG 2000 ( $\diamond$ ) +  $\text{Na}_2\text{SO}_4 + \text{H}_2\text{O} + 5\%$  (w/w) of  
1806  $[\text{C}_2\text{mim}]\text{Cl}$  (red),  $[\text{C}_4\text{mim}]\text{Cl}$  (green),  $[\text{C}_6\text{mim}]\text{Cl}$  (yellow). The lines correspond to the  
1807 systems composed with PEG and salt only. Data taken from <sup>[69]</sup>.

1808 **Fig. 7:** Chemical structures of HBAs and HBDs commonly used in DES preparation.

1809 **Fig. 8:** Phase diagrams of DES-based ABS. **A)** Representation of the binodal curves of  
1810 acetic acid:ChCl- and glucose:ChCl-based ABS as a function of the ChCl concentration for  
1811 DES composed of acetic acid:ChCl at 1:2 ( $\bullet$ ), 1:1 ( $\bullet$ ), and 2:1 ( $\bullet$ ) molar ratio and for  
1812 DES composed of glucose:ChCl at 1:2 ( $\blacksquare$ ), 1:1 ( $\blacksquare$ ), and 2:1 ( $\blacksquare$ ) molar ratio. The line  
1813 corresponds to the system composed with PPG and ChCl only. Data taken from <sup>[28-29]</sup>. **B)**  
.814 Representation of the binodal curves of sugars:ChCl- and alcohols:ChCl-based ABS as a  
.815 function of ChCl concentration for DES composed of ChCl and fructose ( $\blacksquare$ ), glucose ( $\blacksquare$   
.816 ), sucrose ( $\blacksquare$ ), xylose ( $\blacksquare$ ), sorbitol ( $\blacksquare$ ) at 1:1 molar ratio and for DES composed of ChCl  
.817 and *n*-propanol ( $\blacktriangledown$ ), ethanol ( $\blacktriangledown$ ), 1,2-propanediol ( $\blacktriangledown$ ), glycerol

1818 (▼), ethylene glycol (▼) at 1:1 molar ratio.. The line corresponds to the system composed  
1819 with  $K_2HPO_4$  and ChCl only. Data taken from <sup>[30-31, 83]</sup>.

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